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Genetic Predisposition to Breast and Ovarian Cancer in Eastern Finnish Population

Doctoral dissertation

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ABSTRACT

Twenty-five percent of the genetic susceptibility to breast cancer is explained by the susceptibility genes known so far. The aims of this study were to investigate the genetic background of familial breast/ovarian cancer and sporadic breast cancer in Eastern Finnish population.

The frequency and type of germ-line mutations in known high-risk breast/ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, were evaluated in 36 Eastern Finnish breast/ovarian cancer families. In addition, the autosomes were screened for linkage disequilibrium-based association using 435 microsatellite markers to identify new chromosomal regions and genes on them as genetic risk factors for sporadic breast cancer in a case-control set from the Eastern Finnish population.

Germ-line mutations in *BRCA1* and *BRCA2* genes were observed in 19.4 % of the studied Eastern Finnish breast/ovarian cancer families. The observed Eastern Finnish mutation spectrum differs from those observed in the Northern and Southern parts of the country as only two of the eight most common Finnish founder mutations were detected in the Eastern Finnish families. In addition, a novel *BRCA2* 4088insA mutation was found in one family. It has not been found elsewhere in Finland and it appears to be associated with a favourable clinical outcome of breast and ovarian cancer patients but is also highly penetrant.

In the two-staged autosome-wide scan altogether 27 microsatellite markers in 16 chromosomes showed association with breast cancer. In the haplotype analysis three chromosomal regions, 3p26, 11q23 and 22q12-q13 were further suggested as candidate locations for breast cancer associated genes. Breast cancer associated risk factors potentially locate also in the vicinity of single associated markers.

The associated region on chromosome 22q12-q13 was further studied using 10 SNP markers. Significant association was detected with one SNP located in the intronic sequence of *TMPRSS6* gene encoding matriptase-2. The heterozygous genotype and the minor allele were associated with increased risk of breast cancer. Matriptase-2 is a serine protease with functions in processes occurring in both normal and pathological conditions, including cancer progression. Thus, *TMPRSS6* is a potential novel candidate for breast cancer risk affecting gene.

In the AI analysis of 22q12-q13 abundant AI was detected more centromeric than previously reported, further supporting the existence of a tumour suppressor gene or genes in this region.

This study revealed that the proportion of mutations in high-penetrance genes predisposing to familial breast/ovarian cancer in Eastern Finland is similar to other parts of the country, but the mutation spectrum is different. This study also shows that several low-penetrance risk factors for sporadic breast cancer, some of which may be population specific, exist in the Eastern Finnish population.

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Kuopio, October 2007

Jaana Hartikainen

ABBREVIATIONS

3β-HSD	3-betahydroxysteroid dehydrogenase
17β-HSD	17-betahydroxysteroid dehydrogenase
ADH	alcohol dehydrogenase
ADH1B , *ADH1B*	alcohol dehydrogenase type 2, alcohol dehydrogenase type 2 gene
ADH1C, *ADH1C*	alcohol dehydrogenase type 3, alcohol dehydrogenase type 3 gene
ADPRT	poly(ADP-ribose) polymerase 1 gene
AI	allelic imbalance
AIB1/SRC-3	amplified in breast cancer 1/steroid receptor coactivator 3 protein
*AIB1=NCOA3*3	amplified in breast cancer 1 gene
Alu	family of short interspersed nuclear elements
ApaI	restriction fragment length polymorphism in *VDR* gene
APC	adenomatous polyposis coli
APEX1=APE1, APE1	apurinic endonuclease gene, apurinic endonuclease
AR, *AR*	androgen receptor, androgen receptor gene
A-T	Ataxia-telangiectasia
ATM, ATM	ataxia-telangiectasia mutated, gene and protein
ATR	ataxia-telangiectasia and rad3-related
BAP1	BRCA-interacting protein 1
BARD1, *BARD1*	BRCA1-associated ring domain 1 protein, BRCA1-associated ring domain 1 protein gene
BCCIPα/β	BRCA2-interacting protein alpha and beta
BER	base-excision repair
BIC	Breast Cancer Information Core
BIR	break-induced replication
BLM	DNA helicase recq-like type 2
bp	base pairs
BRAF35	BRCA2-associated factor, 35-kD
BRC	internal repeats in *BRCA2* gene
BRCA1, BRCA1	breast cancer 1, gene and protein
BRCA2, BRCA2	breast cancer 2, gene and protein
BRCT	BRCA1 carboxy-terminal
BRIP1=BACH1	BRCA1-interacting protein 1 gene
BsmI	restriction fragment length polymorphism in *VDR* gene
BTF2-TFIIH	basal transcription factor complex
CA125	cancer antigen 125
CACNG2	voltage-dependent calcium channel gamma-2 subunit gene
CASP8	caspase 8 gene
CCND1	cyclin D1 gene
Cdc25A	cell division cycle 25A tyrosine phosphatase
cdk4, cdk6	cyclin dependent kinase 4, cyclin dependent kinase 6
CDKN1A, *CDKN1B*, *CDKN2A*	cyclin-dependent kinase inhibitor 1A, 1B and 2A genes
cDNA	complementary DNA (deoxyribonucleic acid)
Cds1	Schizosaccharomyces pombe gene
CE	catechol oestrogens

CE-SQ, CE-Q	catechol oestrogen semi-quinone, catechol oestrogen quinone
CETN2	centrin 2 gene
CHEK2, *CHEK2*, *CHK2*	checkpoint kinase 2, checkpoint kinase 2 gene
Chk2	mouse checkpoint kinase 2 gene
CI	confidence interval
cM	centi-Morgan
CMF/CNF	adjuvant chemotherapy conatining cyclophosphamide, methotrexate, 5-fuorouracil and mitoxantrone
c-MYC	human homolog of avian v-myc myelocytomatosis viral oncogene
COMT, *COMT*	catechol-O-methyltransferase, catechol-O-methyltransferase gene
CS	Cowden syndrome
CSA, *CSB*	excision-repair cross-complementing group 8 and 6 genes
CSF2RB	granulocyte-macrophage colony-stimulating factor receptor beta gene
CSGE	conformation-sensitive gel electrophoresis
CYPs, CYP450	cytochrome P450 subfamily enzymes
CYP1A1, *CYP1A1*	cytochrome P450 subfamily I polypeptide 1/cytochrome P450A1, cytochrome P450 subfamily I polypeptide 1/cytochrome P450A1 gene
CYP1A2	cytochrome P450 subfamily I polypeptide 2 gene
CYP1B1, *CYP1B1*	cytochrome P450 subfamily I polypeptide 1/cytochrome P4501B1, cytochrome P450 subfamily I polypeptide 1/cytochrome P4501B1 gene
CYP11A	cytochrome P450 subfamily XIA polypeptide 1
CYP17, *CYP17*	steroid 17-α hydroxylase, steroid 17-α hydroxylase gene
CYP19, *CYP19*	aromatase/oestrogen synthetase, aromatase gene
DDB1, *DDB2*	DNA damage-binding protein 1 and 2 genes
DHEA	dehydroepindrosterone
DHPLC	denaturing high-pressure liquid chromatography
DMC1	human homolog of yeast disrupted meiotic cDNA 1 gene
Dmnk	Drosophila melanogaster gene
DNA	deoxyribonucleic acid
DNA-PK, *DNA-PK*	DNA-activated protein kinase catalytic subunit, DNA-activated protein kinase catalytic subunit gene
dNTP	deoxyribonucleotide triphosphate
DSB, DSDB	double-stranded DNA break
DSS1	deleted in split-hand/split-foot 1 region
E1, E2	oestrone, oestradiol
E2F1	E2F transcription factor 1
EDH17B2	17-betahydroxysteroid dehydrogenase I gene
EDTA	ethylenediamine tetra-acetic acid
EM	expectation maximization algorithm
EMSY	protein ecoded by EMSY gene
ER	oestrogen receptor
ERBB2 =HER2/neu	human homolog to avian v-erb-b2 erythroblastic leukaemia viral oncogene
ERCC1	excision-repair complementing defective in Chinese hamster 1 gene

ERCC4=XPF	excision-repair complementing defective in Chinese hamster 4 gene
ESR1, *ESR2*	oestrogen receptor alpha and beta genes
EtBr	ethidium bromide
FA	Fanconi anemia
FA-N	Fanconi anemia complementation group N
FANCD2, FANCD2	Fanconi anemia complementation group D2, gene and protein
FDH	Finnish disease heritage
FEN1	flap structure-specific endonuclease 1 gene
FGFR2, *FGFR2*	fibroblast growth factor receptor 2, fibroblast growth factor receptor 2 gene
FIGO	International Federation of Gynaecology and Obstetrics
FokI	restriction fragment length polymorphism in *VDR* gene
GH, *GH1*	growth hormone, growth hormone gene
GHRH	growth hormone releasing hormone gene
GHRHR, *GHRHR*	growth hormone releasing hormone receptor, growth hormone releasing hormone receptor gene
GHRL	growth hormone secretagogue receptor ligand gene
GHSR	growth hormone secretagogue receptor gene
GSH	glutathione
GST	glutathione S-transferase
GSTM1, *GSTM3*, *GSTP1*, *GSTT1*	glutathione S-transferase M1, M3, P1 and T1 genes
GSTM1	glutathione S-transferase M1
GSTM3	glutathione S-transferase M3
GSTP1	glutathione S-transferase P1
GSTT1	glutathione S-transferase T1
GTF2H1-4	general transcription factor IIH polypeptide 1-4 genes
GWS	genome-wide scan
H19	H19 gene
HA	heteroduplex analysis
HBCC	hereditary breast and colorectal cancer
HDAC1, HDAC2	histone deacetylase 1, histone deacetylase 2
HER2/NEU=ERBB2	human homolog to avian v-erb-b2 erythroblastic leukaemia viral oncogene
HLA	major histocompatibility complex
hMSH3	human homolog 3 of E. coli MutS gene
HNPCC	hereditary nonpolyposis colon cancer syndrome
hPMS1, *hPMS2*	human homologs of S cerevisiae postmeiotic segregation increased 1 and 2 genes
HR	homologous recombination
HRAS1	v-ha-ras Harvey rat sarcoma viral oncogene homolog gene
htSNP	haplotype-tagging single nucleotide polymorphism
HWE	Hardy-Weinberg equilibrium
ICAM5	intercellular adhesion molecule 5 gene
ID4	inhibitor of DNA binding 4
IGF	insulin-like growth factor
IGF-I, IGF-II	insulin-like growth factor I and II genes
IGF-I, IGF-II	insulin-like growth factor I and II
IGF-IR, IGF-IIR	insulin-like growth factor I and II receptors

IGFBP	insulin-like growth factor binding protein
IGFBP3	insulin-like growth factor binding protein 3 gene
IGFBP3	insulin-like growth factor binding protein 3
IL2RB	interleukin 2 receptor beta gene
kb	kilobases
KBCP	Kuopio Breast Cancer Project
KCTD17	potassium channel tetramerization domain gene
kD	kilo Dalton
KU70	thyroid autoantigen, 70-kD
LD	linkage disequilibrium
LFS, LFL	Li-Fraumeni syndrome, Li-Fraumeni-like
LIG1, *LIG3*	DNA ligase 1 and DNA ligase 3 genes
LIG4	DNA ligase 4 gene
LKB1=STK11	serine-threonine protein kinase 11 gene
LOD	logarithm of odds
LOH	loss of heterozygosity
LSP1	lymphocyte-specific protein gene
Mb	mega bases
MLH1, *MLH1= hMLH1*	human homolog of E. coli MutL, human homolog of E. coli MutL gene
MMR	mismatch repair
MnSOD, *MnSOD= SOD2*	manganese superoxide dismutase, manganese superoxide dismutase gene
MPO, *MPO*	myeloperoxidase, myeloperoxidase gene
MPST	mercaptopyruvate sulfurtransferase
MRE11	homolog of S. cerevisiae meiotic recombination 11 gene
mRNA	messenger ribonucleic acid
MSH2, *MSH2= hMSH2*	human homolog 2 of E. coli MutS, homolog 2 of E coli MutS gene
MSH6, *hMSH6*	human homolog 6 of E. coli MutS and homolog 6 of E coli MutS gene
MUTYH	human homolog of E. coli MutY gene
NADPH	nicotinamide adenine dinucleotide phosphate
NAT1, *NAT1*	N-acetyltransferase 1, N-acetyltransferase 1 gene
NAT2, *NAT2*	N-acetyltransferase 2, N-acetyltransferase 2 gene
NBR2	neighbour of BRCA1 gene 2
NBS1, *NBS1*	nibrin, nibrin gene
NCBI	National Center for Biotechnology Information
NCF4	neutrophil cytosilic factor 4 gene
NER, GG-NER	nucleotide-excision repair, global genome nucleotide-excision repair
NHEJ	non-homologous end-joining
NLS, NLS1	nuclear localization signal, nuclear localization signal 1
NQO1, *NQO1*	NAD(P)H:quinone oxidoreductase, NAD(P)H:quinone oxidoreductase gene
nt	nucleotide(s)
NTH1	endonuclease III-like 1 gene
NUMA1	nuclear mitotic apparatus protein 1 gene
OB1, OB2, OB3	oligonucleotide/oligosaccharide binding folds 1, 2 and 3
OCCR	ovarian cancer cluster region

OGG1	8-oxoguanine DNA glycosylase gene
OR	odds ratio
PARP-1	poly(ADP-ribose)polymerase 1
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p53	tumour protein p53
p73	tumour protein p73
p185	*ERBB2* gene product, tumour antigen p185
PAHs	polyaromatic hydrocarbons
PALB2, *PALB2*	partner and localizer of BRCA2, partner and localizer of BRCA2 gene
P/CAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen gene
PCR	polymerase chain reaction
PGR	progesterone receptor gene
Plk1	polo-like kinase 1
PML	promyelotic leukaemia protein
POLB	DNA polymerase β gene
poly-A	variable length poly(A) polymorphism in *VDR* gene
POU1F1	POU domain class 1 transcription factor
PR	progesterone receptor
PTEN=MMAC1=TEP1	phosphatase and tensin homolog gene
PTT	protein truncation test
PVALB	parvalbumin gene
RABL4	RAB, member of RAS oncogene family-like 4gene
RAD23A, *RAD23B*	human homolog A and B of yeast Rad23 genes
RAD50	human homolog of S. cerevisiae Rad50 gene
RAD51, RAD51	human homolog of S. cerevisiae Rad51, human homolog of S. cerevisiae Rad51 gene
Rad51	S.cerevisiae Rad51
RAD51B, *RAD51C*, *RAD51D*	human homolog B, C and D of S. cerevisiae Rad51 genes
RAD52	human homolog of yeast Rad52 gene
Rad53	Saccharomyces cerevisiae Rad53 gene
RAD54	human homolog-like of S. cerevisiae Rad54 gene
RB, *RB1*	retinoblatoma protein, retinoblastoma gene
RefSNP	reference single nucleotide polymorphism database
RFC1, RFC2, RFC4	replication factor C1, replication factor C subunit 2 and replication factor C4
RFLP	restriction fragment length polymorphism
RING-finger	a distinct zinc-chelating domain involved in mediating protein-DNA and protein-protein interactions
ROS	reactive oxygen species
RPA, *RPA1-3*	replication protein A1-A3 genes
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SHBG, *SHBG*	sex hormone-binding globulin, sex hormone-binding globulin gene
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SOD2	manganese superoxide dismutase gene

SSA	single-strand annealing
SSCP	single-stranded conformation polymorphism
SST, *SST*	somatostatin, somatostatin gene
SSTR1, *SSTR2*, *SSTR5*	somatostatin receptor 1, 2 and 5 genes
SSTR1-5	somatostatin receptors 1-5
STK15=AURORA2=AURKA=BTAK=ARK1	serine threonine kinase gene
STR	short tandem repeat, microsatellite
SULT	sulfotransferase
SULT1A1, *SULT1A1*	sulfotransferase 1A1, sulfotransferase 1A1 gene
SULT1E1, *SULT1E1*	sulfotransferase 1E1, sulfotransferase 1E1 gene
TaqI	restriction fragment length polymorphism in *VDR* gene
TCR	transcription-coupled repair
TGFβ1, *TGFB1*	transforming growth factor beta, transforming growth factor beta gene
TMPRSS6	transmembrane protease, serine 6, gene
TNFα, *TNFβ*	tumour necrosis factor alpha and beta genes
TNM	tumour-node-metastasis classification
TNRC9=TOX3	TOX high mobility group box family member 3 gene
TP53	tumour protein 53 gene
TST	thiosulphate sulfurtransferase
TTK	phosphotyrosine-picked threonine kinase
UCSC	University of California Santa Cruz
UICC	International Union Against Cancer
USP11	ubiquitin-specific protease 11
UTR	untranslated region
UV	ultraviolet
VDR, *VDR*	vitamin D receptor, vitamin D receptor gene
VEGF, *VEGF*	vascular endothelial growth factor, vascular endothelial growth factor gene
WHO	World Health Organization
VNTR	variable number of tandem repeats
XAB2	XPA-binding protein 2
XPA, *XPG*	xeroderma pigmentosum complementation group A and G genes
XPD=ERCC2	excision-repair complementing defective in Chinese hamster 2 gene
XRCC1	X-ray repair complementing defective in Chinese hamster 1
XRCC1	X-ray repair complementing defective in Chinese hamster 1 gene
XRCC2	X-ray repair complementing defective in Chinese hamster 2 gene
XRCC3	X-ray repair complementing defective in Chinese hamster 3 gene
XRCC4	X-ray repair complementing defective in Chinese hamster 4 gene
XRCC5	X-ray repair complementing defective in Chinese hamster 5 gene
ZNF350=ZBRK1	zinc finger protein 350 gene

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals I-V.

I Hartikainen JM, Kataja V, Pirskanen M, Arffman A, Ristonmaa U, Vahteristo P, Ryynänen M, Heinonen S, Kosma V-M, Mannermaa A: Screening for *BRCA1* and *BRCA2* mutations in Eastern Finnish breast/ovarian cancer families. Clinical Genetics 2007, 72:311-320.

II Hartikainen JM, Mannermaa A, Heinonen S, Kosma VM, Kataja V: A *BRCA2* mutation, 4088insA, in a Finnish breast and ovarian cancer family associated with favourable clinical course. Anticancer Research 2007, in press.

III Hartikainen JM, Pirskanen MM, Arffman AH, Ristonmaa UK, Mannermaa AJ: A Finnish *BRCA1* exon 12 4216-2nt A to G splice acceptor site mutation causes aberrant splicing and frameshift, leading to protein truncation. Human Mutation 2000, 15:120.

IV Hartikainen JM, Tuhkanen H, Kataja V, Dunning AM, Antoniou A, Smith P, Arffman A, Pirskanen M, Easton DF, Eskelinen M, Uusitupa M, Kosma VM, Mannermaa A: An autosome-wide scan for linkage disequilibrium-based association in sporadic breast cancer cases in Eastern Finland: three candidate regions found. Cancer Epidemiology, Biomarkers & Prevention 2005, 14:75-80.

V Hartikainen JM, Tuhkanen H, Kataja V, Eskelinen M, Uusitupa M, Kosma VM, Mannermaa A: Refinement of the 22q12-q13 breast cancer-associated region: evidence of *TMPRSS6* as a candidate gene in an Eastern Finnish population. Clinical Cancer Research 2006, 12:1454-1462.

This thesis includes also unpublished data. The original papers in this thesis have been reproduced with the permission of the publishers.

CONTENTS

1. INTRODUCTION

Breast cancer is the most common cancer among women in industrialised countries. Approximately one in ten women in Finland will develop breast cancer during her lifetime. Currently more than 4000 new breast cancer cases are diagnosed yearly and the number is increasing (Finnish Cancer Registry). At year 2005 ovarian cancer was the ninth most common cancer in the Finnish female population accounting 424 new ovarian carcinomas (Finnish Cancer Registry).

Many risk factors (hormonal, environmental) for breast cancer are known but most of the genetic background and molecular mechanisms still remain to be elucidated. Although mutations in *BRCA1* (Hall et al. 1990, Miki et al. 1994) and *BRCA2* (Wooster et al. 1994, Wooster et al. 1995) genes are known to confer a high lifetime risk of breast cancer, together with the other susceptibility genes so far identified, *ATM* (Swift et al. 1987, Easton 1994, Athma et al. 1996), *CHEK2* (Bell et al. 1999, CHEK2-Breast Cancer Consortium 2002), *TP53* (Easton 1999), *PALB2* (Rahman et al. 2007), and *BRIP1* (Seal et al. 2006), these genes explain only ≤ 25 % of the familial aggregation of breast cancer (Antoniou and Easton 2006).

Several linkage studies in large family material have been conducted but other *BRCA1/2*-like, high-risk, high-penetrance breast cancer susceptibility genes have not been found. Therefore, the yet unidentified genes presumably are numerous and confer a moderate risk (Thompson and Easton 2004). In a study investigating the genetic models of the non-*BRCA1/2* familial clustering of breast cancer the findings suggest that several common, low-penetrance genes may account for the residual familial aggregation of breast cancer (Antoniou et al. 2002). It is most likely that low-penetrance genes for breast cancer susceptibility are present in the general population (Pharoah et al. 1997).

In Finland, 26 different *BRCA1* and *BRCA2* mutations have been found among female and male breast cancer and ovarian cancer patients (Vehmanen et al. 1997a, Vehmanen et al. 1997b, Roth et al. 1998, Huusko et al.1998, Sarantaus et al. 2000, Syrjäkoski et al. 2000, Sarantaus et al. 2001a, Sarantaus et al. 2001b, Pääkkönen et al. 2001, Eerola et al. 2001, Vahteristo et al. 2001a, Syrjäkoski et al. 2004a). Fourteen of

these, seven in each gene, are considered as founder mutations as they are recurrent and account for the majority of all detected *BRCA1* and *BRCA2* mutations in Finland. Some of these mutations are unique to the Finns. Due to the population history the Finns are genetically isolated homogeneous population and founder effects can be seen in several autosomal recessive diseases and cancer predisposition syndromes (Norio et al. 1973, Nyström-Lahti et al. 1994, Moisio et al. 1996). This effect has been observed among *BRCA1* and *BRCA2* mutations also and it has an impact on diagnostics as well (Sarantaus et al. 2000). The proportion of *BRCA1* and *BRCA2* mutations among Southern, Western and Northern Finnish breast/ovarian cancer families has been studied but in Eastern Finnish population it has not been reported yet.

Linkage disequilibrium (LD) -based genetic association studies are suitable tools for detecting low-penetrance susceptibility genes that likely interact with environmental and lifestyle factors as well as with other genetic factors to cause disease. Young, isolated populations (e.g. the Eastern Finns) may provide more help by reducing the genetic heterogeneity. LD, or allelic association, is based on the assumption that the affected share a genetic variant/mutation, which is so close to the marker that the probability of a recombination event to occur between them is minimal. In young (15-25 generations) isolated populations the number of meioses is relatively low and LD is thought to extend further than in older populations (Ophoff et al. 2002). LD analysis has been used to discover genes for several diseases of the Finnish disease heritage e.g. neuronal ceroid lipofuscinosis and diastrophic dysplasia (Järvelä 1991, Hästbacka et al. 1992), as well as in other isolated populations and complex diseases like severe bipolar disorder and congenital muscular dystrophy (Ophoff et al. 2002, Toda et al. 1996). In detection of susceptibility genes for complex disease also SNP haplotypes have been successfully used in the Finnish population (Laitinen et al. 2004).

In the present study the proportion of *BRCA1* and *BRCA2* germ-line mutations was evaluated in breast/ovarian cancer families of Eastern Finnish origin. Also, to find new genetic medium or low-penetrance risk factors for sporadic breast cancer, the autosomes were screened utilizing linkage disequilibrium-based association and 435 microsatellites in a breast cancer case-control set from Eastern Finland. One of the associated regions

(22q12-q13) was further investigated by SNP association analysis to identify the putative breast cancer related gene.

2. REVIEW OF THE LITERATURE

2.1 Breast cancer

2.1.1 Incidence of breast cancer

Breast cancer is the most common cancer among women in industrialised countries. Each year over one million new cases are diagnosed in the world and it is the leading cause of cancer death (Globocan 2002). The incidence is highest in Northern America, Western and Northern Europe, and Australia/New Zealand (Globocan 2002). In Finland the proportion of breast cancer is approximately one third (31.8 % in 2005) of all female cancers (Finnish Cancer Registry). One in ten women in our country will be affected with breast cancer at some point in her life. Every year more than 3 500 new cases are diagnosed and the number is increasing, the number of new cases in 2005 already being 4 027 (Finnish Cancer Registry). The age-adjusted incidence of breast cancer was 86.7 per 100 000 person years in the year 2005 in Finland (Finnish Cancer Registry). In 2005 the number of breast cancer deaths was 804, i.e. the age-adjusted mortality rate was 14.7 per 100 000 person years. The age-adjusted mortality rate has remained rather unchanged since the 1950's (14 - 16 / 100 000 person years). Thus, a relative decline in breast cancer mortality has been observed following the initiation of the population based mammography screening programme in 1987 (Finnish Cancer Registry 2002 and 2004, Botha et al 2003). Male breast cancer in Finland is rare, accounting for less than 1 % of all breast cancers annually (Finnish Cancer Registry 2002).

2.1.2 Epidemiology and risk factors for breast cancer

Oestrogens have an important role in the development and progression of breast cancer. The life-time exposure to oestrogen is related to the risk of developing the disease (Pike et al 1979). Thus, the risk of breast cancer rises throughout a woman's lifetime (Finnish Cancer Registry 2002). Breast cancer before the age of 25 years is rare, except in certain familial cases (Finnish Cancer Registry 2002). Risk factors for breast cancer are early age at menarche (MacMahon et al 1982), nulliparity or delayed first childbirth (Layde et al. 1989, Ewertz et al. 1990, Kelsey et al. 1993), short duration of breast-feeding (Layde

et al. 1989, Kelsey et al. 1993), low number of children (Layde et al. 1989, Ewertz et al. 1990), late menopause (Trichopoulos et al. 1972, Collaborative Group on Hormonal Factors in Breast Cancer 1997), postmenopausal obesity (Folsom et al. 1990, Hunter and Willett 1993), extended use of oral contraceptives (Collaborative Group on Hormonal Factors in Breast Cancer 1996) and long-term oestrogen replacement therapy (Collaborative Group on Hormonal Factors in Breast Cancer 1997), which all are surrogates for oestrogen exposure. Higher education and socio-economic status has been found to associate with increased breast cancer risk, which is largely explained by nulliparity, later age at first childbirth and greater use of synthetic hormones (Heck and Pamuk 1997, Pukkala and Weiderpass 1999).

Increased risk of breast cancer is associated with carcinoma of the contralateral breast, a history of benign breast disease e.g. atypical hyperplasia and sclerosing adenosis (Dupont and Page 1985, Carter et al. 1988, Wang et al. 2004, Bernstein et al. 1992), and ionising radiation (Howe and Mclaughlin 1996, Preston et al. 2002, Pukkala et al. 2006). Also cigarette smoke has been associated with an increase in risk for breast cancer (Gram et al. 2005, Johnson 2005) and accumulation of environmental oestrogens, xeno-oestrogens, may have a role in breast cancer aetiology (Davis et al. 1993). Physical inactivity and several dietary factors such as fat or energy intake and high consumption of cooked red meat (Bernstein et al. 1994, Bartsch et al. 1999, Nair et al. 1999, Zheng et al. 1998) as well as high alcohol consumption enhance breast cancer risk (Garfinkel et al. 1988, Smith-Warner et al. 1998). Many epidemiological studies have observed a positive association between adult height and risk of breast cancer, although the explanation for this is not clear (Tretli 1989, Vatten and Kvinnsland 1990, Hunter and Willett 1993). Also race and ethnicity, national origin and geographical location have an influence on breast cancer risk (Ziegler et al. 1993).

Family history of breast/ovarian cancer is a known risk factor. It is recognized that a woman with a first-degree relative with breast cancer has twice the risk of developing the disease herself (Pharoah et al. 1997). Genetic susceptibility accounts for 5-10 % of all cases of breast cancer (Claus et al. 1996). Mutations in *BRCA1* and *BRCA2* genes account for approximately 15 % of the excess familial relative risk of breast cancer (Peto et al. 1999, Anglian Breast Cancer Study Group 2000) and in total the so far

known breast cancer susceptibility genes account for no more than 25 % of the familial aggregation of breast cancer (Easton 1999). In addition, numerous low-penetrance genetic variants exist and these contribute together with environmental and lifestyle factors and with other genes to cause breast cancer (Antoniou et al. 2002).

2.1.3 Prognostic factors in breast cancer

Breast cancer is predominantly a postmenopausal disease and appearance at young age, <35 years, is related to aggressive disease and reduced survival (Shannon and Smith 2003). Tumour stage is the most important prognostic factor in breast cancer and it is the basis for selecting patients for different treatment strategies. Breast cancer stage is assessed according to the TNM classification of the UICC and it involves tumour size, lymph node status and distant metastases (International Union Against Cancer 2002). Histological grade, based on tubule formation, nuclear pleiomorphism, and the number of mitoses, has independent prognostic value in breast cancer (WHO 2003). Expression of oestrogen and progesterone receptors in the tumour tissue predicts a good response to hormonal therapy of breast cancer (Clarke et al. 2004), whereas *ERBB2* (*HER2*/*neu*) gene amplification and/or protein overexpression in the tumour tissue is associated with poor prognosis (Ross et al. 2003). Gene expression profiling of breast tumours has enabled classification of tumours by their gene expression patterns, and provided insights into development of new potential diagnostic and prognostic markers (Perou et al. 2000, van't Veer et al. 2002, Sorlie et al. 2003, Chang et al. 2005).

2.2 Ovarian cancer

2.2.1 Incidence of ovarian cancer

Ovarian cancer is among the most common cancers in women worldwide with over 200,000 new cases annually. The incidence rates are highest in Europe, North America and Australia/New Zealand (Globocan 2002). In Finland, ovarian cancer was the ninth most common of female cancers accounting for 424 new cases in year 2005 (Finnish Cancer Registry). In 2005 the ovarian cancer incidence was 8.4 and mortality rate 5.0

per 100 000 person years. In addition to ovarian carcinomas, approximately 110 borderline tumours are diagnosed annually. The estimation of the number of new cases of ovarian cancer in 2006 is 507. (Finnish Cancer Registry).

2.2.2 Epidemiology and risk factors for ovarian cancer

The risk factors for ovarian cancer include genetic and environmental (hormonal and lifestyle) factors, many of them shared with breast cancer risk. Ageing and family history of ovarian or breast cancer increase the risk. Also hormone replacement therapy has been associated with increased risk. The number of full-term pregnancies and use of oral contraceptives are inversely correlated with risk, and late age at menarche, early age at menopause and breastfeeding modestly reduce the risk. Also tubal sterilisation has been associated with reduction in risk. The effects of obesity, physical activity and fertility treatment are unclear. (Bertone-Johnson 2005).

It is established that at least 10 % of all epithelial ovarian cancers are hereditary, with the *BRCA* genes contributing to at least 90 % of these cases and small percentages attributable to hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (*MSH2* and *MLH1* genes) and perhaps some yet to be discovered susceptibility gene (Boyd 2003). It is presumed that genetic and environmental factors affect the penetrance of *BRCA* gene mutations for ovarian cancer but very few factors have been identified to date. One such factor is the *HRAS1* variable number of tandem repeat (VNTR). The rare alleles have been suggested to increase the ovarian (but not breast) cancer risk in *BRCA1* mutation heterozygotes (Phelan et al. 1996). *HRAS1* VNTR rare alleles have been shown to increase the relative risk of ovarian cancer in general population also (Weitzel et al. 2000).

2.2.3 Prognostic factors in ovarian cancer

The most important prognostic factor in ovarian cancer is the FIGO (International Federation of Gynaecology and Obstetrics) stage. Stage I tumours are limited to ovaries, stage II to ovaries with pelvic extension, stage III to ovaries with peritoneal implants and stage IV to ovaries with distant metastasis. (Cancer Committee of the FIGO, 1986).

Histological grade describing the tumour differentiation is also associated with prognosis (WHO 2003). The best-known and well-described serum marker for ovarian cancer is the CA125 antigen (Whitehouse and Solomon 2003).

2.3 Familial breast cancer and high risk genes

Breast cancer attributable to family history of the disease has been reported to account for 5-10 % of all cases of breast cancer (Claus et al. 1996). A number of genes exist with a proven high penetrance to familial breast cancer when mutated. High-risk breast and ovarian cancer susceptibility genes, *BRCA1* (Hall et al. 1990, Miki et al. 1994) and *BRCA2* (Wooster et al. 1994, Wooster et al. 1995), are estimated to explain ~15-20 % of the excess familial relative risk of breast cancer (Table 1) (Peto et al. 1999, Anglian Breast Cancer Study Group 2000). Other loci for breast cancer susceptibility have also been identified, including *PTEN*, *TP53*, *ATM*, *CHEK2*, *BRIP1* and *PALB2*, but these are of lower frequency in the general population (Table 1) (Easton 1999, CHEK2-Breast Cancer Consortium 2002, Seal et al. 2006, Rahman et al. 2007). Moderate family history of breast cancer has been shown to independently predict breast cancer risk without associated *BRCA1* or *BRCA2* mutations (Pharoah et al. 1997). Overall, breast

Table 1. Breast cancer risks conferred by inactivating mutations in known susceptibility genes.

Gene	Overall relative risk of breast cancer	Contribution to		References
		Breast cancer (%)	Familial risk (%)	
BRCA1	>30 at <40 years 14 at >60 years	1-2	*	Thompson and Easton 2004, Antoniou et al. 2003
BRCA2	11	1-2	*	Thompson and Easton 2004, Antoniou et al. 2003
ATM	2.37	0.86	†	Renwick et al. 2006
CHEK2	2.34	0.7	0.5†	CHEK2 Breast Cancer Case-Control Consortium, 2004
PALB2	2.3	0.23	0.24†	Rahman et al. 2007
BRIP1	2.0 (3.5 at <50)	0.2	†	Seal et al. 2006

*Collectively these account for ~15-20 % of the overall familial risk of breast cancer (Peto et al. 1999, Anglian Breast Cancer Study Group 2000).
†Collectively these account for ~2.3 % of the overall familial relative risk (Rahman et al. 2007).

cancer is approximately twice as common in women with an affected first-degree relative; this risk increases with the number of affected relatives and is greater for women with relatives affected at a young age (Collaborative Group on Hormonal Factors in Breast Cancer 2001). However, in families with both breast and ovarian cancers, the aggregation of these two cancers appears to be explained by *BRCA1/BRCA2* mutation-carrier probability (Claus et al. 1998).

2.3.1 *BRCA1* and *BRCA2* genes

In 1990 linkage to marker *D17S74* on chromosome 17q21 was detected in a study using 23 families with 146 breast cancer cases (Hall et al. 1990). In most of the families the typical features for hereditary breast cancer were seen; early age of onset, bilateral cases and male breast cancer. Later the *BRCA1* (Breast Cancer 1 gene) gene was identified and cloned on 17q21 (Miki et al. 1994). In another linkage study using 22 families with at least one male breast cancer case strong negative LOD score values were obtained suggesting that male breast cancer is not linked to the *BRCA1* locus on 17q (Stratton et al. 1994). An analysis using 15 families with no linkage to *BRCA1* locus revealed linkage to 13q12-q13, which was presumed to be the locus for *BRCA2* (Breast Cancer 2 gene) (Wooster et al. 1994). *BRCA2* gene was cloned on chromosome 13q12 in 1996. Both genes encode charged and exceptionally large proteins. (Tavtigian et al. 1996). (Figure 1).

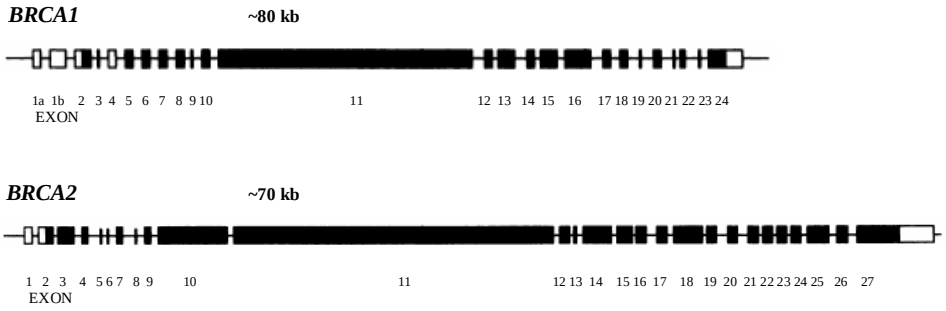


Figure 1. Genomic structure of the *BRCA1* and *BRCA2* genes. *BRCA1* gene spans over 80 kb of genomic sequence consisting of 24 exons and *BRCA2* approximately 70 kb with 27 exons (Miki et al. 1994, Tavtigian et al. 1996). Modified from Håkansson et al. 1997.

2.3.2 The BRCA1/2 proteins and their functions

2.3.2.1 Protein structure and functional motifs

Twenty-two of the 24 exons of *BRCA1* gene encode an 1863 amino-acid-protein (Miki et al. 1994). The protein is expressed in several tissues e.g. in ovary, testis and mammary gland epithelial cells (Miki et al. 1994). The 3418 amino-acid BRCA2 protein is encoded by 25 of the 27 exons of *BRCA2* gene (Tavtigian et al. 1996). The BRCA2 protein does not share sequence homology with any other protein (Wooster et al. 1995, Tavtigian et al. 1996). Like BRCA1, the BRCA2 protein is expressed in several tissues, including mammary gland, spleen, ovary, lung, testis and thymus (Tavtigian et al. 1996). Although BRCA1 and BRCA2 proteins have only little resemblance to each other or other proteins of known function, they contain structural motifs that give clues of their biochemical functions. (Figure 2).

In its amino-terminal region BRCA1 contains a zinc binding RING-finger motif that is found in regulatory proteins and appears to be involved in protein-protein interactions (Miki et al. 1994, Saurin et al. 1996). This motif has a role in e.g. the interaction of BARD1 (BRCA1-associated RING domain protein) and BAP1 (BRCA1-interacting protein 1) with BRCA1, as well as in BRCA1 homodimer formation (Wu et al 1996, Jensen et al. 1998, Brzovic et al. 1998). Two nuclear localization signals (NLS) locate in BRCA1 exon 11 but presumably only one (NLS1) of them is needed for the nuclear localization of BRCA1 (Chen et al. 1996, Thakur et al. 1997). The carboxy terminal-region of BRCA1 contains a conserved BRCT (BRCA1 C-terminal) domain that acts as a protein-binding site and interacts with multiple transcription factors (Koonin et al. 1996), and a region that has been shown to have transcriptional activation potential (Chapman & Verma 1996). (Figure 2).

In the C-terminal region of BRCA2 protein locates three oligonucleotide/oligo-saccharide binding folds (OB1, OB2, OB3) that bind DSS1 and single-stranded DNA (Yang et al. 2002a). This provided a structural and biochemical basis for understanding the loss of recombination-mediated double-strand break repair in BRCA2-associated cancers (Yang et al. 2002a). Also an the amino-terminal region within exon 3 of BRCA2 has been shown to have transcriptional activation potential (Milner et al. 1997). In its' central part, in exon 11, BRCA2 contains eight conserved sequence motifs, the

BRC repeats that interact directly with RAD51 protein (Bork et al. 1996, Bignell et al. 1997, Wong et al. 1997). Within the final 156 residues in the carboxy-terminal of BRCA2 resides two nuclear localization signals (NLS) that are required for the nuclear localization and proper function of the BRCA2 protein (Spain et al. 1999). (Figure 2). In addition, both *BRCA1* and *BRCA2* have been suggested to have properties of granins as they include a motif similar to the granin consensus at the C-terminus of the protein (Jensen et al. 1996).

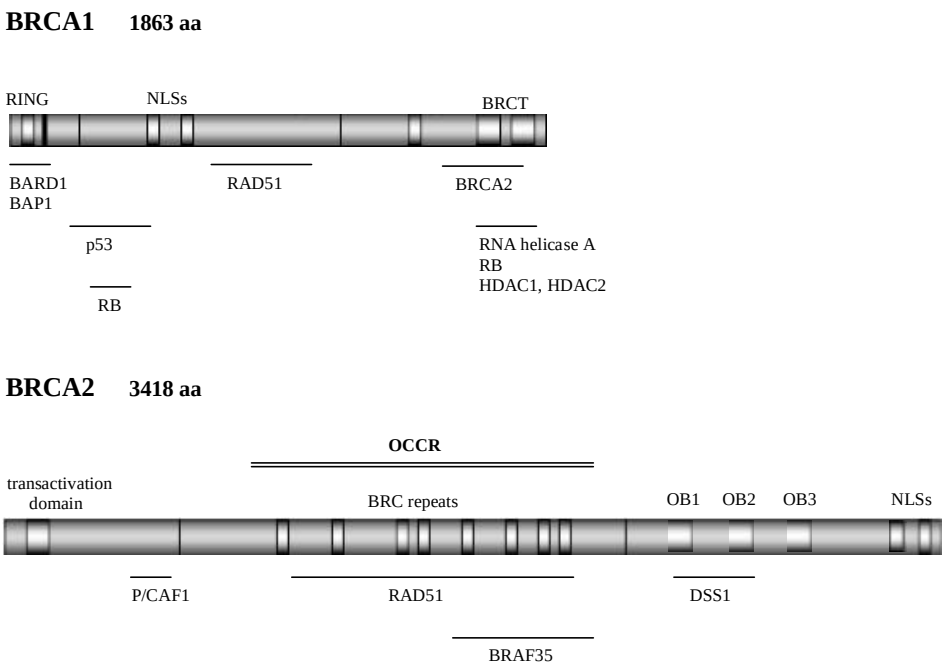


Figure 2. Structural domains and functional motifs of BRCA1 and BRCA2 proteins. The borders of exon 11 are shown with black lines. NLS=nuclear localization signal; OB1, OB2, OB3=oligonucleotide/oligo-saccharide binding folds; OCCR=ovarian cancer cluster region. Some of the most important interacting proteins are shown below the proteins. See text for details. Modified from Borg 2001.

2.3.2.2 Proposed functions

BRCA1 and BRCA2 proteins interact with a number of regulatory proteins and they seem to have multiple fundamental functions. BRCA1 interacts directly and indirectly with numerous molecules, including tumour suppressors, oncogenes, DNA damage

repair proteins, cell cycle regulators, transcriptional activators and repressors, e.g. BRCA2, BARD1, ATM, ATR, CHEK2, BLM, MSH2, MSH6, MLH1 and RFC1-RFC2-RFC4 complex (Wang et al. 2000, Borg 2001, Welcsh and King 2001). BRCA2 interacts with Rad51, BRCA1, DSS1, EMSY, P/CAF, BRAF35, Plk1, USP11, FANCD2 and G, androgen receptor (AR), BCCIPα/β and PALB2 (Xia et al. 2006). Both BRCA1 and BRCA2 proteins localize to the nucleus of dividing cells and work in pathways that are required for the maintenance of chromosome structure (Chen et al. 1996, Thakur et al. 1997, McAllister et al. 1997, Scully et al. 1997a, Chen et al. 1998a, Welsch et al. 2000). BRCA1 and BRCA2 have major roles in maintaining genomic integrity e.g. through tumour suppression, transcription regulation, cell proliferation and differentiation, cell cycle control and participating in DNA repair. Recently it has been reported that BRCA2 is the Fanconi anaemia D1 protein and, as such, plays a key role in complex series of nuclear events that promote DNA crosslink repair (Howlett et al. 2002, Taniguchi and D'Andrea 2006). Also BRCA1 has been proposed to be involved in the Fanconi anaemia-BRCA pathway (Garcia-Higueira et al. 2001, D'Andrea and Grompe 2003). In their role as tumour suppressors BRCA1 and BRCA2 behave as caretakers, suppressing genome instability as they are essential for preserving the chromosome structure (Xu et al. 1999, Tirkkonen et al. 1997, Patel et al. 1998, Gretarsdottir et al. 1998).

BRCA1 and *BRCA2* are considered as gatekeeper tumour suppressor genes that are involved in multiple tumour types, and as caretaker genes (Schutte et al. 1995, Thompson et al 1995, Holt et al. 1996, Wang et al. 2002, Kinzler and Vogelstein 1997). Gatekeepers directly regulate the growth of tumours by inhibiting growth or promoting death (Kinzler and Vogelstein 1997). *BRCA* genes have been shown to suppress breast cancer cell growth (Thompson et al. 1995, Holt et al. 1996, Wang et al. 2002). According to Knudson's double hit hypothesis the inactivation of a tumour suppressor (or gatekeeper) gene needs two mutations, usually one germ-line and one somatic, and leads to tumour development (Knudson 1971). Mutations in caretaker genes do not directly result in tumour formation but instead they cause genetic instability which leads to increased mutations in the genome and eventually to tumour formation (Kinzler and Vogelstein 1997). Tumours arising in women carrying a single germ-line mutant

BRCA1 or *BRCA2* allele exhibit LOH at this locus, losing the wild-type allele and retaining the mutant copy of the gene, implying that BRCA1 and BRCA2 proteins function as tumour suppressors (Smith et al. 1992, Collins et al. 1995, Cornelis et al. 1995). How these proteins exert the tumour suppressor functions is incompletely understood.

BRCA1 and BRCA2 participate in the biological response to DNA damage, which involves the activation of cell cycle checkpoints and the recruitment of the machinery for DNA repair. Failure to activate these checkpoints or DNA repair following DNA damage manifests as increased sensitivity to genotoxic agents (Patel et al. 1998, Sharan et al. 1997, Xu et al. 2001, Scully et al. 1997b). In addition to the role in DNA repair after damage caused by exposure to exogenous agents, BRCA1 and BRCA2 proteins function in DNA damage response after endogenous damage that arise during processes such as DNA replication or transcription (Yarden and Brody 1999, Milner et al. 1997, Chapman et al. 1996, Scully et al. 1997c, Fuks et al. 1998, Bochar et al. 2000, Venkitaraman 2002, Welcsh et al. 2000, Scully and Livingston 2000). Although both are essential for error-free homologous recombination (HR) BRCA1 and BRCA2 have distinct roles in DSB repair. Disruption of BRCA2 prevents efficient homologous recombination (HR) but single-stranded annealing (SSA) and non-homologous end-joining (NHEJ), which are more error-prone, are used (Xia et al. 2001, Moynahan et al. 2001, Tutt et al. 2001), whereas in BRCA1 deficient cells SSA and HR appear to be decreased and NHEJ predominates as the mechanism for DSB repair (Moynahan et al. 1999). The major role of BRCA2 is through control of the RAD51 recombinase (Chen et al. 1998b, Wong et al. 1997, Davies et al. 2001, Pellegrini et al. 2002, Yang et al. 2002a, Venkitaraman 2002, Pellegrini and Venkitaraman 2004), while BRCA1 performs a distinct and more general function as a link between the sensing/signaling and effector components of the mammalian response to DNA damage, helping to ensure that the ensuing response is appropriate to the initiating lesion (Wang et al. 2000, Zhong et al. 1999, Cortez et al. 1999, Lee et al. 2000, Venkitaraman 2002). Chromosomal instability provoked by *BRCA* deficiency is the result of incorrect routing of DSB processing down inappropriate pathways, rather than the failure of repair per se (Venkitaraman 2002).

2.3.3 Cancer risks conferred by mutations in the *BRCA* genes

In a recent analysis of pooled pedigree data unselected for family history with breast or ovarian cancer from 22 studies, the average cumulative risks in *BRCA1* mutation carriers by age 70 years were 65 % for breast cancer and 39 % for ovarian cancer. The corresponding estimates for *BRCA2* were 45 % and 11 % (Antoniou et al. 2003). In high risk families the risks are somewhat higher, especially for breast cancer in *BRCA2* mutation carriers; ~85 % by age 70 and the risk for ovarian cancer ~27 % (Easton et al. 1995, Ford et al. 1998). The risk in *BRCA1*-mutation carriers by age 70 is ~70 % for breast cancer and ~40 % for ovarian cancer (Easton et al. 1995, Ford et al. 1998). The risk for subsequent breast or ovarian cancer is also high in breast cancer patients carrying *BRCA1* and *BRCA2* mutations (Easton et al. 1995, Breast Cancer Linkage Consortium 1999). The cumulative risk for male breast cancer in *BRCA2* mutation carriers has been estimated to be 3-6 % by age 70 years (Easton et al. 1997, Thompson and Easton 2001). Although the majority of families with male breast cancer are connected with germ-line *BRCA2* mutations, also *BRCA1* is suggested to contribute a larger fraction to male breast cancer than previously thought (Couch et al. 1996, Frank et al. 2002).

In addition to the high risks of breast and ovarian cancers, *BRCA2* mutations may be associated with increased risks of several other cancers. Statistically significant increases in risks were observed for prostate cancer, pancreatic cancer, stomach cancer, gallbladder and bile duct cancer and malignant melanoma (Breast Cancer Linkage Consortium 1999). The overall increases in risks for other than breast and ovarian cancer are quite small, except for prostate cancer in *BRCA2* mutation carriers where the cumulative risk has been estimated to be 10-34 % by age 80 years (Breast Cancer Linkage Consortium 1999, Thompson and Easton 2001). In *BRCA1* mutation carriers the overall increased risk of cancer at sites other than breast and ovary is small and is observed in women but generally not in men. *BRCA1* mutations may confer increased risks of other abdominal cancers in women and increased risks of pancreatic cancer in men and women (Thompson et al. 2002a).

2.3.3.1 Variation in cancer risks conferred by *BRCA* mutations

Depending on the *BRCA* mutation different risks of breast, ovarian, and other cancers, and possibly different probabilities for particular tumour types or tumour progression are conferred (Gayther et al. 1995, Gayther et al. 1997a, Scott et al. 2003). Different hypotheses have been introduced as the biological explanation for the variation in cancer risk by mutation location but none of them has been confirmed (Thompson and Easton 2001, Thompson et al. 2002b, Ware et al. 2006). The risk of cancer among the carriers may also differ considerably, due to e.g. chance, genetic, hormonal, dietary, and environmental modifying factors (Antoniou et al. 2000, Antoniou et al. 2002, Thompson and Easton 2004).

A lower risk of ovarian cancer in relation to breast cancer risk has been reported to be associated with mutations in the 3' end of the *BRCA1* gene, whereas the mutations in the central part of the gene confer a lower breast cancer risk than other mutations (Risch et al. 2001, Gayther et al. 1995, Thompson et al. 2002b). For *BRCA2*, mutations in the central part of the gene, in a region known as the ovarian cancer cluster region, OCCR (Figure 2), at nucleotides 3035-6629, appear to be associated with a lower breast cancer risk and higher ovarian cancer risk than other *BRCA2* mutations (Gayther et al. 1997a, Thompson and Easton 2001). Later, the OCCR was slightly modified on statistical grounds to comprise nucleotides 3059-4075 and 6503-6629 (Thompson and Easton 2001). Also splice-site mutations in both *BRCA1* and *BRCA2*, as well as substitutios in the *BRCA2* gene have been suggested to confer higher risks than substitutions in the *BRCA1* gene (Scott et al. 2003).

Possible genetic factors that modify the effect of *BRCA* gene mutations, besides *BRCA* genes themselves, include rare *HRAS1* alleles and a polymorphism in the *RAD51* gene (Phelan et al. 1996, Levy-Lahad et al. 2001, Wang et al. 2001a).

2.3.4 Clinical features of *BRCA* gene mutation-associated cancers

Several histopathological and clinical features differ among *BRCA1*, *BRCA2* and sporadic breast cancers. The pathological characteristics of *BRCA2*-associated breast tumours are less clear than for *BRCA1*, and overall their behaviour seems to be more similar to those in sporadic cases. (Table 2).

Table 2. Clinical features of *BRCA1* and *BRCA2* mutation-associated breast and ovarian cancers.

***BRCA1*-associated**	***BRCA2*-associated**	**References**
Breast carcinomas diagnosed at younger age	Breast carcinomas diagnosed at younger age	Hall et al. 1990, Easton et al. 1993, Miki et al. 1994, Wooster et al. 1994, Ford et al. 1994, Easton et al. 1995, Boyd et al. 2000, Risch et al. 2001
Bilateral cancer more common	Bilateral cancer more common	
Ovarian carcinomas diagnosed at younger age		
Often ER- and PR-	Predominantly ER+ and PR+	Johannsson et al. 1997, Loman et al. 1998, Lakhani et al. 2000
High histological grade	Evidence of higher average grade (effect weaker than for *BRCA1*)	Marcus et al. 1996, Breast Cancer Linkage Consortium 1997, Johannsson et al. 1997, Lakhani et al. 1998
High mitotic count		
More often highly proliferating and aneuploid		
Overrepresentation of medullary or atypical medullary tumours		Breast Cancer Linkage Consortium 1997
Distinguished gene expression patterns in tumours	Distinguished gene expression patterns in tumours	Hedenfalk et al. 2001
A trend towards worse survival in breast cancer found in some studies	Less data but not likely to be very different from sporadic Worse survival also reported	Foulkes et al 1997, Verhoog et al. 1998, 2000, Loman et al. 2000, Stoppa-Lyonnet et al. 2000, Goode et al. 2002a, Robson et al. 2003, Brekelmans et al. 2006
Ovarian carcinomas typically of serous histology, moderate to high grade and advanced stage Mucinous subtype underrepresented		Rubin et al. 1996, Boyd et al. 2000, Moslehi et al. 2000, Risch et al. 2001, Lakhani et al. 2004
A trend towards better survival in ovarian cancer (due to enhanced susceptibility to chemotherapy)	Little data available, better survival when combined analysis with *BRCA1*	Rubin et al. 1996, Pharoah et al. 1999, Boyd et al. 2000, Ben David et al. 2002, Narod and Boyd 2002, Cass et al. 2003

Significantly different groups of genes are expressed by breast cancers with *BRCA1* mutations and breast cancers with *BRCA2* mutations, and sporadic breast tumours, and thus, these three groups can be distinguished from each other. This supports further the molecular difference between cancers with underlying germ-line mutations in *BRCA1* and *BRCA2* genes as well as cancers without such mutations. (Hedenfalk et al. 2001). Also ovarian tumours segregate into "*BRCA1*-like" and "*BRCA2*-like" subgroups according to the gene expression profiles. Mutations in *BRCA1* and *BRCA2* may lead to carcinogenesis through distinct molecular pathways that also appear to be involved in

sporadic cancers. (Jazaeri et al. 2002).

2.3.5 Mutation spectrum and founder mutations in the *BRCA* genes

More than 1 500 different mutations or polymorphisms have been identified in the *BRCA1* gene and almost 1 900 in the *BRCA2* gene (BIC, February 2006). The majority of mutations are frameshift mutations caused by small insertions or deletions, or alterations affecting the splice-site (BIC). Most of these mutations hamper the ability of the plausible tumour-suppressor or caretaker gene to block the development of cancer.

A mutational hotspot in the *BRCA1* or *BRCA2* gene has not been described but frequent founder mutations have been discovered in some populations, of which the Icelandic and Ashkenazi Jewish populations are the most studied. In Iceland the preponderance of a single *BRCA2* 999del5 mutation has been observed (Gudmundsson et al. 1996, Thorlacius et al. 1996). (Table 3). It is estimated to account for up to 76 % of families with multiple cases of female breast cancer and/or male breast cancer (Thorlacius et al. 1996). In addition to the 999del5 mutation in the *BRCA2* gene, only one rare mutation in the *BRCA1* gene has been identified in the Icelandic population (Bergthorsson et al. 1998).

Table 3. Predominant founder mutations in *BRCA1* and *BRCA2* genes.

Population	Founder mutation	Freq. in population	Breast cancer risk in female carriers	Reference
Icelandic	*BRCA2* 999del5	0.6 % (1/170)	37 % at age 70	Johannesdottir et al. 1996, Thorlacius et al. 1997, Thorlacius et al. 1998
Ashkenazim	*BRCA1* 185delAG	~1 %	36 % (life-time)	Fodor et al. 1998
	BRCA1 5382insC	0.25 %		Fodor et al. 1998
	BRCA2 6174delT	~1 %	36 % (life-time)	Fodor et al. 1998

The Ashkenazi Jews have three ancestral founder mutations, 185delAG and 5382insC in *BRCA1* (Simard et al. 1994) and 6174delT in *BRCA2* (Tavtigian et al. 1996) (Table 3). These mutations account for almost all the *BRCA1/2* mutations found in this population. The life-time risk for breast cancer among Ashkenazi Jewish carriers (regardless of age of onset or family history of cancer) of the *BRCA1* 185delAG or

BRCA2 6174delT mutations is approximately 3 times the overall risk for the general population (Fodor et al. 1998). Different estimations of higher risks of breast and ovarian cancer among the carriers of the Ashkenazi Jewish founder mutations in any population have been also published (Struewing et al. 1997, King et al. 2003, Antoniou et al. 2005).

In addition to the Ashkenazi Jews, the 5382insC mutation and another *BRCA1* mutation, 4153delA, are frequent among breast/ovarian cancer families of Eastern European, e.g. Russian, Hungarian, Latvian and Polish origin (Neuhausen et al. 1996, Gayther et al. 1997b, Ramus et al. 1997a, Csokay et al. 1999, Gorski et al. 2000). Patients being heterozygous carriers of two Ashkenazi Jewish founder mutations, 185delAG/6174delT and 5382insC/6174delT, have been detected (Ramus et al. 1997b, Friedman et al. 1998). The observations suggest that the phenotypic affects of double heterozygosity for *BRCA1* and *BRCA2* germ-line mutations are not cumulative (Friedman et al. 1998). A patient of a non-Jewish ancestry carrying both *BRCA1* and *BRCA2* mutations, G2508T/3295insA, as well as a patient homozygous for *BRCA1* 2800delAA mutation have also been observed (Liede et al. 1998, Boyd et al. 1995).

In the Dutch population large Alu-mediated deletions in the *BRCA1* gene are recurrent and are among the founder mutations (Petrij-Bosch et al. 1997, Puget et al. 1997, Swensen et al. 1997). Alu-mediated duplications of the *BRCA1* gene have been found (Puget et al. 1999), and a genomic deletion in the *BRCA2* gene in a Swedish breast/ovarian cancer family has also been published (Nordling et al. 1998).

Recurrent founder mutations are being identified in a growing number of populations as the data on *BRCA1* and *BRCA2* mutations accumulates. Founder effect is also evident in Finland (Vehmanen et al. 1997a, Huusko et al. 1998, Sarantaus et al. 2000).

2.3.6 *BRCA* mutations in Finland

In Finland 15 different *BRCA1* and 11 different *BRCA2* mutations have been found among female and male breast cancer and ovarian cancer patients so far (Vehmanen et al. 1997a, Vehmanen et al. 1997b, Roth et al. 1998, Huusko et al.1998, Sarantaus et al. 2000, Syrjäkoski et al. 2000, Sarantaus et al. 2001a, Sarantaus et al. 2001b,

Pääkkönen et al. 2001, Eerola et al. 2001, Vahteristo et al. 2001a, Syrjäkoski et al. 2004a). Fourteen of these, seven in each gene, are recurrent and are considered as founder mutations in Finland as they account for the majority of all detected mutations. Some of these mutations are unique to the Finns. The most recurrent mutation in *BRCA2* is the Icelandic founder mutation 999del5 (Table 4). Interestingly, the Icelandic and Finnish *BRCA2* 999del5 mutations could have an ancient common origin and in addition to Iceland and Finland the 999del5 mutation has not been reported in other Nordic countries (Barkardottir et al. 2001). Large genomic alterations have not been found in *BRCA1* or *BRCA2* gene in the Finnish population (Lahti-Domenici et al. 2001, Laurila et al. 2005, Karhu et al. 2006).

Table 4. The four most recurrent founder mutations in the *BRCA1* and *BRCA2* gene in Finland.

Gene	Mutation
BRCA1	4216-2nt A→G
	3604delA
	3744delT/3745delT
	4446C→T
BRCA2	999del5
	7708C→T
	8555T→G
	9346-2ntA→T

In the previous studies conducted in 88 Northern and 100 Southern Finnish families *BRCA1/2* mutations were found in 12.5 % and 21 % of the families, in 8-43 % depending on the strength of family history and being the highest in true high-risk families with both breast and ovarian cancers. *BRCA1* mutations were observed in 7 % and 10 % of studied breast/ovarian cancer families. The corresponding figures for *BRCA2* were 6 % and 11 %. (Huusko et al. 1998, Vehmanen et al. 1997a). The proportion of *BRCA2* mutations was slightly higher than *BRCA1* mutations. The highest (80 %) frequencies of *BRCA* mutations in Finland have been observed in families with both breast and ovarian cancer and early-onset breast cancer, while in families with later-onset breast cancer only, the mutation frequency has been the lowest, 1.5 % (Vahteristo et al. 2001a). Among 1035 unselected Finnish breast cancer cases *BRCA2* mutations were considerably more frequent with the frequency

of 1.4 %, than *BRCA1* mutations with the frequency of 0.4 % (Syrjäkoski et al. 2000). In Finnish ovarian carcinoma families *BRCA1/2* founder mutations were found in 26 % (Sarantaus et al. 2001a) and *BRCA2* mutations were slightly more frequent than *BRCA1* mutations. However, among unselected ovarian carcinoma patients *BRCA1/2* founder mutations account for an approximate 5 % of ovarian carcinomas (Sarantaus et al. 2001b) and *BRCA1* mutations are more frequent than *BRCA2* mutations.

2.3.7 Common polymorphisms and *BRCA* genes in sporadic breast/ovarian cancer

The most common polymorphisms which cause amino acid substitutions in the *BRCA1* gene, Gln356Arg, Pro871Leu, Glu1038Gly and Ser1613Gly, do not make a significant contribution to breast and ovarian cancer risk (Dunning et al. 1997, Wenham et al. 2003, Auranen et al. 2005). However, the homozygous 356Arg genotype was suspected to be protective against breast cancer (Dunning et al. 1997). Cox and co-workers (2005a) reported a haplotype in the *BRCA1* gene that was associated with a small (18 %) increase in risk of sporadic breast cancer. However, the functional variants responsible for the association were unclear. They also reported no association with Gln356Arg and breast cancer. In another recent multiethnic study with breast cancer case-control material no evidence for significant associations between risk of sporadic breast cancer and common variation in *BRCA1* was found (Freedman et al. 2005).

The only common nonsynonymous SNP in the *BRCA2* gene is Arg372His (N372H). In a joint analysis of British, German and Finnish population the rare homozygotes (HH) were first associated with a 30 % increase in risk of breast cancer, with the strongest association observed in women <45 years of age (Healey et al. 2000a). This was also observed in an Australian study (Spurdle et al. 2002) but not in studies conducted in the US (Freedman et al. 2004, Cox et al. 2005b, Garcia-Closas et al. 2006). The rare homozygous genotype HH has also been shown to associate with increased risk of ovarian cancer in Australian and British population (Auranen et al. 2003), whereas in a US study no association was detected (Wenham et al. 2003). In the largest collaborative study conducted so far, no significant association was found

between increased risk of breast cancer and the HH homozygotes, and only a marginally statistically significant interaction of N372H with age (Breast Cancer Association Consortium 2006).

Somatic inactivating mutations in *BRCA1* and *BRCA2* gene are very rare and thus do not play a major role in the tumorigenesis of sporadic breast or ovarian cancer (Futreal et al. 1994, Meravjer et al. 1995, Miki et al. 1996, Lancaster et al. 1996, Teng et al. 1996, Weber et al. 1996). However, allelic deletions have been observed in sporadic breast and ovarian cancers in both genes, which imply that *BRCA* genes may play roles as tumour suppressor genes in sporadic breast and ovarian cancers also (Futreal et al. 1994, Takahashi et al. 1995, Beckmann et al. 1996, Gras et al. 2001, Yang-Feng et al. 1993). Also inactivation and reduction in expression levels of *BRCA1* in sporadic breast and ovarian cancers have been detected (Thompson et al. 1995, Taylor et al. 1998, Wilson et al. 1999, Esteller et al. 2000, Russell et al. 2000). The silencing of the *BRCA1* gene in primary breast and ovarian carcinomas occurs epigenetically by promoter hypermethylation, especially in the presence of LOH (Dobrovic and Simpfendorfer 1997, Baldwin et al. 2000, Rice et al. 1998, Bianco et al. 2000, Esteller et al. 2000). Hypermethylation of the *BRCA2* promoter region has not been observed in breast cancer (Collins et al. 1997) and only very rarely in ovarian cancers (Hilton et al. 2002). Instead, it has been suggested that *BRCA2* is silenced by overexpression of EMSY (Hughes-Davies et al. 2003). EMSY binds to exon 3 of *BRCA2* and thereby suppresses the transcriptional activity of BRCA2 (Hughes-Davies et al. 2003).

Other possible mechanisms leading to low BRCA1 expression are regulation of protein stability, allele specific expression, p53 or ID4 mediated expression, activation of the negative regulatory site in the first intron, loss of activation by transcription factors or activation of NBR2 expression (reviewed in Mueller and Roskelley 2003). BRCA1 protein has also been observed to be mislocalised to cytoplasm (Chen et al. 1995). However, the extent to which *BRCA1* and *BRCA2* contribute to the pathogenesis of sporadic breast and ovarian cancer remains unclear as *BRCA1* and *BRCA2* may affect through other more indirect mechanisms.

2.3.8 Other genes involved in familial breast cancer

In addition to the hereditary breast and ovarian cancer syndrome caused by mutations in the *BRCA1* and *BRCA2* genes, breast cancer occurs as a component of other inherited cancer predisposition syndromes. Germ-line mutations in these predisposing genes are however, very rarely seen in breast cancer patients without manifestations of other features of the syndromes. Thus, the contribution of these genes (*PTEN*, *TP53*, *ATM*, *LKB1*) to familial aggregation of site-specific breast cancer is marginal. Low-penetrance genes accounting for the familial aggregation of breast cancer have been also identified. These include *CHEK2* and a recently identified *PALB2*.

2.3.8.1 *PTEN* and Cowden syndrome

Cowden syndrome (CS) is an autosomal dominant disorder, characterised by the development of hamartomas and benign tumours in multiple organs, e.g. skin, intestine, thyroid and breast, and an increased risk for breast, thyroid and endometrial carcinomas. Mutations in the tumour suppressor *PTEN* gene (also known as *MMAC1* or *TEP1*), on chromosome 10q22-q23, are present in over 80 % of Cowden syndrome families (Nelen et al. 1996, Liaw et al. 1997, Eng 2003). Truncating *PTEN* mutations in Cowden syndrome families are associated with cancer and cause a 25-50 % lifetime risk of breast cancer in women and the age at diagnosis of breast cancer in patients with CS is approximately 10 years younger than in sporadic cases (Starink et al. 1986, Longy and Lacombe 1996). The risk of male breast cancer might also be increased (Marsh et al. 1998, Fackenthal et al. 2001). No mutations in *PTEN* gene have been detected in breast cancer families without CS features (Tsou et al. 1997, Carroll et al. 1999, Lauge et al. 1999). In sporadic breast cancer *PTEN* mutations or polymorphisms seem not to play a significant role either (FitzGerald et al. 1998, de Jong et al. 2002, Haiman et al. 2006).

2.3.8.2 *TP53* and Li-Fraumeni syndrome

The *TP53* gene is located on chromosome 17p13 (McBride et al. 1986). The p53 protein encoded by *TP53* is a 53-kD phosphoprotein that has four distinct domains: an N-terminal transactivation domain, a central DNA-binding domain, a tetrametization domain and a C-terminal regulatory domain (Soussi and May 1996). p53 is a

transcription factor and it is activated and stabilized in response to different forms of cellular stress, e.g. ionizing radiation, chemotherapeutic drugs and UV light, via post-translational modifications and protein-protein interactions, after which it transactivates or transrepresses the genes involved in cell-cycle control, DNA repair, apoptosis, and the formation of blood vessels (Vogelstein et al. 2000). p53 has been named as the "guardian of the genome" due to the multiple roles of the wild-type p53 protein in controlling the cell-cycle and proliferation (Lane 1992).

Germ-line mutations in the *TP53* gene cause Li-Fraumeni syndrome (Malkin et al. 1990). Li-Fraumeni syndome (LFS) is a rare autosomal dominant disorder, characterised by an increased risk of soft tissue and osteosarcomas, leukaemias, brain tumours, adrenocortical carcinomas, and breast cancer (Li and Fraumeni 1969, Li et al. 1988). The risk of developing breast cancer before the age of 45 is 18-fold higher among LFS females compared to the general population (Garber et al. 1991). Germline *TP53* mutations have been found in approximately 70 % of LFS and 25 % of LFL (Li-Fraumeni-like) families (Varley et al. 1997, Eng et al. 1997). Germ-line mutations in the *TP53* gene have been estimated to account for less than 1 % of breast cancer but recent studies suggest a proportion higher than that, especially among cases with disease onset at the age 30 years or younger (Börresen et al. 1992, Sidransky et al. 1992, Lalloo et al. 2003). Germ-line mutations in *TP53* have also been found in Finnish breast cancer families meeting the LFS/LFL criteria (Huusko et al. 1999, Rapakko et al. 2001).

Germ-line mutations of *TP53* in familial, non-LFS or non-LFL, early-onset, or bilateral breast cancer cases are rare (Börresen et al. 1992, Lidereau and Soussi 1992, Sidransky et al. 1992, Balz et al. 2002, Ohayon et al. 2005, Lalloo et al. 2006). An Arg72Pro polymorphism is located in a proline-rich region of the *TP53* gene that is required for the growth suppression activity of p53 (Walker et al. 1996). After the initial finding of increased risk of breast cancer in homozygous carriers of the proline allele Arg72Pro has been examined in numerous studies for the possible role in breast cancer risk (Sjalander et al. 1996). The largest case-control studies so far have found no association between Arg72Pro and breast cancer risk (Wang-Gohrke et al. 2002, Tommiska et al. 2005, Breast Cancer Association Consortium 2006).

2.3.8.3 *ATM*

Ataxia-telangiectasia (A-T) is a rare autosomal recessive degenerative disorder caused by biallelic mutations of the *ATM* gene on chromosome 11q22-q23 (Savitsky et al. 1995). A-T is characterized by progressive cerebellar ataxia, a weakened immune system, hypersensitivity to ionizing radiation, highly increased susceptibility to malignancies, primarily of lymphoid origin, and distinctive dilated blood vessels in the eyes and skin. The ATM protein encoded by *ATM* is a large serine-threonine kinase that is activated in response to DNA damage by ionizing radiation (Canman et al. 1998, Kastan and Lim 2000). Upon activation ATM phosphorylates a wide array of downstream targets that regulate cell cycle checkpoints, apoptosis, and DNA repair (Kastan and Lim 2000, Bakkenist and Kastan 2003). ATM also regulates several tumour suppressors, including p53, BRCA1 and CHEK2 (Canman et al. 1998, Banin et al. 1998, Khanna et al. 1998, Cortez et al. 1999, Matsuoka et al. 1998).

Studies based on relatives of A-T patients have shown that heterozygous carriers of variant *ATM* are clinically unaffected but are shown to be at increased risk of cancer, especially the female carriers who have a two- to seven-fold increased risk of breast cancer (Swift et al. 1987, Pippard et al. 1988, Borresen et al. 1990, Swift et al. 1991, Easton 1994, Athma et al. 1996, Olsen et al. 2001, Thompson et al. 2005). In contrast, mutation screening of *ATM* in breast cancer case-controls sets outside A-T families have produced mixed results and have suggested allelic heterogeneity of *ATM* and that only a specific class of variants contribute to breast cancer risk in heterozygotes (FitzGerald et al. 1997, Teraoka et al. 2001, Gatti et al. 1999, Chenevix-Trench et al. 2002). Recently it has been shown in a UK study using familial breast cancer case-control population that A-T-causing biallelic *ATM* mutations are breast cancer susceptibility alleles in monoallelic carriers outside A-T families, and the combined *ATM* mutation prevalence and contribution to breast cancer incidence is similar to *CHEK2* 1100delC; both confer an estimated two-fold risk of breast cancer (Renwick et al. 2006). Association of some of the missense variants (7271T>G, Ser49Cys) has been observed in other large case-control studies also (Bernstein et al. 2006, Stredrick et al. 2006). In the Breast Cancer Association Consortium study the Ser49Cys was not significantly associated with overall breast cancer risk, but a modest association was not excluded either, and this

SNP increased the risk of PR positive breast cancer (Cox et al. 2007). Results from studies of association between common polymorphisms (SNPs) in *ATM* and risk of breast cancer have been controversial so far (Angele et al. 2003, Tamimi et al. 2004a, Lee et al. 2005a). In the Finnish population germ-line mutations in *ATM* have an apparent but overall limited contribution to familial and sporadic breast cancer predisposition outside A-T families (Pylkäs et al. 2007).

2.3.8.4 Checkpoint kinase 2, *CHEK2* (*CHK2*)

2.3.8.4.1 *CHEK2* gene and protein function

The cloning of the gene encoding the checkpoint kinase 2, CHEK2, was reported by five laboratories in 1998/1999 (Matsuoka et al. 1998, Blasina et al. 1999, Chaturvedi et al. 1999, Brown et al. 1999, Tominaga et al. 1999). The *CHEK2* (also known as *CHK2*) gene locates on chromosome 22q12.1 and spans 50 kb of genomic DNA. Fourteen exons encode a 543-amino-acid protein with 83 % identity to mouse Chk2 and 34 % identity to *Drosophila* Dmnk (Matsuoka et al. 1998). CHEK2 is a also the human homolog of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 protein kinases that have crucial roles in controlling cell-cycle checkpoints after DNA damage and stalled replication (Matsuoka et al. 1998, Blasina et al. 1999, Chaturvedi et al. 1999, Brown et al. 1999). At the protein level the structure is dominated by several evolutionally conserved elements (Matsuoka et al. 1998, Bartek et al. 2001).

CHEK2 is a G2 checkpoint protein kinase that plays a critical role in maintaining genomic stability (DNA repair) and coordinates cell cycle responses. In mammalian cells CHEK2 is activated in response to replication blocks and DNA damage caused by UV light or ionising radiation (Matsuoka et al. 1998). ATM phosphorylates CHEK2 which in turn phosphorylates other key cell cycle proteins, including BRCA1 and p53 (Matsuoka et al. 1998, Chehab et al. 2000, Lee et al. 2000, Shieh et al. 2000). In addition to ATM-dependent activation CHEK2 is activated in an ATM-independent, ATR-dependent manner (Matsuoka et al. 1998, Chaturvedi et al. 1999, Blasina et al. 1999, Hirao et al. 2002). CHEK2 has been also shown to regulate apoptosis through a p53-independent pathway by phosphorylating the promyelocytic leukemia protein, PML

(Yang et al. 2002b). Furthermore, CHEK2 has been reported to regulate also E2F1 transcription factor activity in response to the DNA-damaging agent etoposide (Stevens et al. 2003). On the other hand, E2F1 expression results in increase in CHEK2 protein levels and it may be essential for p53 activation and apoptosis induction (Rogoff et al. 2004). Ectopic expression of E2F1 induces the ATM-dependent phosphorylation of CHEK2 and stimulates the kinase activity of CHEK2 (Powers et al. 2004). NBS1 is also required for the induction of CHEK2 phosphorylation induced by E2F1 (Powers et al. 2004). Moreover, CHEK2 plays a critical role in the pro-apoptotic transcription factor p73 protein induction following DNA damage and modulation of E2F1 makes an important contribution (Urist et al. 2004). A recent study suggests that a mitotic checkpoint kinase TTK participates in the regulation of DNA damage by functioning upstream of CHEK2 and phosphorylating it (Wei JH et al. 2005). DNA-PK is also suggested to be involved in activation of CHEK2 in response to DNA damage (Li and Stern 2005).

Checkpoint mechanisms and DNA-repair machinery are needed for temporary halting of cell-cycle progression in proliferating cells, in order to provide time for repair and prevent fixation and propagation of harmful mutations (Hartwell and Weinert 1989, Elledge 1996, Weinert 1998, Walworth 2000, Bartek et al. 2001). When DNA damage occurs, activated CHEK2 targets several cell cycle modulators. These events ultimately lead to cell cycle arrest, DNA repair, or programmed cell death. Failures in the checkpoint machinery can result in developmental malformations, embryonic lethality or the accumulation of mutations that could potentially lead to genetic disease, including cancer (Hartwell 1992, Hartwell and Kastan 1994, Weinert 1997, Dasika et al. 1999). CHEK2 mutations have been detected in tumours and it has been suggested that constitutive activation of ATM-CHEK2 pathway occurs at pre-invasive stages of major types of human tumours, including colon, breast and lung carcinomas (Bartkova et al. 2005).

2.3.8.4.2 *CHEK2* germ-line mutations and risk of breast cancer

The first germ-line *CHEK2* mutations were reported as early as in 1999 in both sporadic and hereditary human cancers. Germ-line mutations in *CHEK2,* including 1100delC,

were detected in Li-Fraumeni syndrome patients without *TP53* mutations (Bell et al. 1999). The findings suggested that *CHEK2* is a tumour suppressor gene conferring predisposition to sarcoma, breast cancer and brain tumours, and provided a link between the central role of p53 inactivation in human cancer and the well-defined G2 checkpoint in yeast (Bell et al. 1999). The 1100delC mutation was also detected in Finnish families suggestive of Li-Fraumeni syndrome (Vahteristo et al. 2001b). Recent studies have confirmed that *CHEK2* is mutated in a subset of familial breast cancers (CHEK2-Breast Cancer Consortium 2002, Vahteristo et al. 2002, Meijers-Heijboer et al. 2003, Oldenburg et al. 2003). Both truncated and missense mutants of CHEK2 have lost their kinase activity i.e. the ability to interact with, and efficiently phosphorylate, substrates such as Cdc25A and p53 (Falck et al. 2001a, Falck et al. 2001b, Wu et al. 2001). It has been suggested that the range of cancers associated with mutations of the *CHEK2* may be much greater than previously thought and they may increase the risk of cancer in many different organs (Cybulski et al. 2004). However, this was not confirmed in a large multicenter study (Thompson et al. 2006). Significant association to ovarian cancer has not been detected either (Baysal et al. 2004).

The *CHEK2* 1100delC was shown to be a relatively common low-penetrance allele conferring susceptibility to breast cancer (CHEK2-Breast Cancer Consortium 2002, Vahteristo et al. 2002, CHEK2 Breast Cancer Case-Control Consortium 2004) (Table 5). The 1100delC variant allele has been estimated to result in an approximately 2-fold increase of breast cancer risk in women and a 10-fold increase of risk in men. In *BRCA1* or *BRCA2* mutation carriers the 1100delC allele did not confer increase in cancer risk, possibly because of functional redundancy (CHEK2-Breast Cancer Consortium 2002, CHEK2 Breast Cancer Case-Control Consortium 2004). The estimated 10-fold risk for male breast cancer or the high carrier frequency among males (CHEK2-Breast Cancer Consortium 2002) has not been confirmed in other studies and is likely to be smaller (Syrjäkoski et al. 2004b, Neuhausen et al. 2004, Sodha et al. 2004, Ohayon et al. 2004).

Vahteristo and co-workers (2002) suggested that *CHEK2* acts as a low-penetrance tumour-suppressor gene in breast cancer and that it makes a significant contribution to familial clustering of breast cancer - including families with only two affected relatives, which are more common than families that include larger numbers of affected women.

Table 5. Observed prevalence of the *CHEK2* 1100delC mutation.

	Observed frequency of *CHEK2* 1100delC mutation in					
Population	Controls	Breast cancer cases in *BRCA1/2* mutation neg. families	Breast cancer cases with family history	Breast cancer cases in families with *BRCA1/2* mutations	Population-based breast cancer cases	**References**
UK, Netherlands, North America	1.1 %	5.1 %		1 %	1.4 %	CHEK2-Breast Cancer Consortium 2002
Finland	1.4 %	5.5 %	3.1 %		2.0 %	Vahteristo et al. 2002
UK, Netherlands, Finland, Germany, Australia	0.7 %				1.9 %	CHEK2 Breast Cancer Case-Control Consortium 2004
Netherlands	2.8 %	11.4 %				Oldenburg et al. 2003
Germany	0.9 %		1.5 %, 1.4 %		0.45 %, 0.8 %	Dufault et al. 2004, Rashid et al. 2005
Spain	0 %	0 %				Osorio et al. 2004
Czech and Slovak			0.2 %		0.4 %	Kleibl et al. 2005
Italy	0 %	0 %	0.11 %			Caligo et al. 2004
Australia			0.6 %			Jekimovs et al. 2005
US	0.4 %, 0.5 %		1.1 %		1.1 %, 1.2 %	Mateus-Pereira et al. 2004, Friedrichsen et al. 2004
Ashkenazi Jews	0.3 %		1.0 %			Offit et al. 2003

In the collaborative analysis the prevalence of 1100delC was somewhat greater in women reporting a first-degree relative with breast cancer and also some evidence was seen that the prevalence in cases decreased with increasing age at diagnosis (CHEK2 Breast Cancer Case-Control Consortium 2004). 1100delC has also been observed to associate with bilateral breast cancer (Vahteristo et al. 2002). A strong association of the *CHEK2* 1100delC with a putative hereditary breast and colorectal cancer phenotype (HBCC) was detected, suggesting a subtype of familial breast cancer (Meijers-Heijboer et al. 2003). It was also suggested that *CHEK2* 1100delC mutation acts in synergy with another as-yet-unknown cancer-susceptibility gene or genes (Meijers-Heijboer et al. 2003, Oldenburg et al. 2003, Johnson et al. 2005).

Variation in 1100delC carrier frequencies in different populations have been observed (CHEK2 Breast Cancer Case-Control Consortium 2004), and it is either absent or occurs in similar frequencies among breast cancer cases and controls in many populations. Thus, it is evident that the contribution of *CHEK2* mutations varies by ethnic groups and from country to country. (Table 5). Other variants in *CHEK2* have not been detected to make a similar contribution to familial breast cancer susceptibility as the 1100delC does (Allinen et al. 2001, Schutte et al. 2003, Dufault et al. 2004, Friedrichsen et al. 2004). Though, significant associations have been found in some study populations where the 1100delC is rare. (Table 6). Common polymorphisms in *CHEK2* are unlikely to confer an increased risk of breast cancer (Kuschel et al. 2003).

Table 6. Observed variants in *CHEK2*, other than 1100delC.

Variant	Population	Association with	References
Ser428Phe	Ashkenzi Jews	familial breast cancer	Shaag et al. 2005
Ile157Thr	Byelorussian	familial breast cancer	Bogdanova et al. 2005
Ile157Thr	Finnish, Polish, German, Byelorussian	sporadic breast cancer	Kilpivaara et al. 2004, Cybulski et al. 2004, 2006a, Bogdanova et al. 2005
deletion of exons 9 and 10	Czech and Slovak, Polish	2-fold increase in risk of breast cancer	Walsh et al. 2006, Cybulski et al. 2007

The carriers of *CHEK2* 1100delC appear to develop ER and PR receptor positive tumours more frequently than non-carriers (de Bock et al. 2004, Schmidt et al. 2007), and an association with lobular histology and a *CHEK2* variant has been suggested (Huzarski et al. 2005, Cybulski et al. 2006). Tumours from 1100delC carriers also tend

to be of higher grade than those of non-carriers (Kilpivaara et al. 2005). Tumours with reduced CHEK2 expression had more often larger primary tumour size compared to tumours with normal expression (Kilpivaara et al. 2005) and CHEK2 expression is often reduced in tumours from patients with germ-line 1100delC (Vahteristo et al. 2002). Otherwise a characteristic *CHEK2* signature in breast tumours has not been discovered yet (Cybulski et al. 2006, Schmidt et al. 2007). A worse disease-free survival has also been suggested for the mutation carriers (de Bock et al. 2004, Schmidt et al. 2007).

2.3.8.5 *LKB1* and Peutz-Jeghers syndrome

Peutz-Jeghers syndrome is a rare autosomal dominant disorder characterised by mucocutaneous pigmentation and gastrointestinal polyposis. In addition to an elevated risk of gastrointestinal cancers, an increased risk of cancers at other sites, such as breast, ovary, uterus, cervix, lung, and testis, has been described. (Giardiello et al. 1987, Tomlinson and Houlston 1997, Giardiello et al. 2000). The cumulative risk of breast cancer by age 70 years has been reported to be as high as 45 % (95 % CI=27-68) (Hearle et al. 2006). The Peutz-Jeghers syndrome is caused by germ-line mutations in the serine-threonine kinase gene *LKB1* (=*STK11*) located on chromosome 19p13 (Hemminki et al. 1998, Jenne et al. 1998). Germ-line mutations in the *LKB1* gene have not been reported in other than Peutz-Jeghers families, and this gene seems to play a role in breast cancer susceptibility only in patients with Peutz-Jeghers syndrome (Chen and Lindblom 2000, de Jong et al. 2002).

2.3.8.6 *PALB2* and Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive syndrome characterized by congenital abnormalities, progressive bone-marrow failure, and cancer susceptibility. (D'Andrea and Grompe 2003). FA is a genetically heterogeneous condition and thus far the disease includes 13 subtypes (complementation groups), 12 of which have been attributed to mutations in distinct genes, suggesting that their gene products function in a common pathway (Taniguchi and D'Andrea 2006, Reid et al. 2007, Patel et al. 2007). The most recently discovered of FA genes is *PALB2* (Reid et al. 2007).

PALB2 (partner and localizer of BRCA2) gene locates on chromosome 16p12 and encodes a recently discovered protein that interacts with BRCA2 (Xia et al. 2006). PALB2 colocalizes with BRCA2 in nuclear foci, promotes its localization and stability in key nuclear structures (e.g. chromatin and nuclear matrix), and enables its recombinational repair and checkpoint functions (Xia et al. 2006). Biallelic *PALB2* germ-line mutations are responsible for a subset of Fanconi anemia (FA-N) cases characterized by a phenotype similar to that caused by biallelic *BRCA2* mutations (Reid et al. 2007, Xia et al. 2007). Monoallelic *PALB2* mutations were also found in individuals with breast cancer from familial breast cancer pedigrees that were negative for *BRCA1* and *BRCA2* mutations. *PALB2* mutations showed an incomplete segregation in affected relatives and were estimated to confer a 2.3-fold increase in breast cancer risk and it was suggested that the risks of breast cancer associated with *PALB2* mutations may be age dependent. The breast cancer population-attributable fraction of *PALB2* mutations in this UK population was estimated to be 0.23 % and the percentage of familial relative risk due to *PALB2* to be 0.24 %. (Rahman et al. 2007). A truncating mutation, being a possible founder mutation, in *PALB2* was also detected in familial and sporadic breast cancer cases and in one prostate cancer family in the Finnish population (Erkko et al. 2007). *PALB2* is the first gene that encodes for a protein that interacts with BRCA2 and confers an increased risk of breast cancer when mutated (Xia et al. 2006, Rahman et al. 2007).

The phenotypes associated with biallelic *BRCA2* and *PALB2* mutations are markedly similar to each other and differ from the other ten known FA genes (Reid et al. 2007, Xia et al. 2007). Biallelic mutations in *BRIP1* cause one of the FA subtypes and heterozygous mutations in *BRIP1* confer an elevated risk of breast cancer (Levran et al. 2005, Seal et al. 2006). However, it is suggested that breast cancer susceptibility is associated with only a subset of Fanconi anemia genes which is consistent with the negative results of mutational screens of other FA genes (Seal et al. 2003). The currently known FA genes that associate with breast cancer, *BRCA2*, *PALB2* and *BRIP1*, are not part of the FA core complex and are the only known FA genes that act downstream of FANCD2 (Rahman et al. 2007).

2.4 Sporadic breast cancer and low-penetrance susceptibility genes

Familial aggregation of breast cancer is thought to account for 5-10 % of all cases of breast cancer (Claus et al. 1996). Currently known high-risk and high-penetrance susceptibility genes confer approximately 20 % of this (Thompson and Easton 2004). However, in twin studies heritable factors are observed even in one fourth of breast cancer cases (Lichtenstein et al. 2000). It is assumed that several common, low to moderate penetrance genes are present in the general population and account also for the residual familial aggregation of breast cancer (Pharoah et al. 1997, Antoniou et al. 2002). The disease causing alleles of high-penetrance genes are relatively rare in the general population, whereas the low-penetrance disease-associated alleles are common. High-penetrance genes predispose to both earlier onset of the disease and multifocal tumours. In contrast to these familial cases, sporadic breast cancers caused by low-penetrance genes usually appear unilaterally and have relatively late age at diagnosis. Low-penetrance genes are likely to interact with environmental and life-style factors as well as with other genes to cause cancer. (Rebbeck 1999). As a result of this etiologic complexity, malignant diseases that do not follow a simple Mendelian pattern of inheritance in a substantial number of families have remained refractory to mapping by linkage. (Le Marchand 2005). It has been observed that subtle effects of some genetic variants may be magnified and only become detectable in the presence of certain exposures (Low et al. 2006). The identification of such low or moderate-penetrance susceptibility alleles is an extensive task. Utilising the candidate-gene approach, polymorphisms in several genes have been tested in breast cancer case-control association studies. In these studies the genes have been selected in pathways of known or suspected breast cancer-associated factors, such as genes affecting the oestrogen levels and metabolism, DNA repair or tumour growth. The genes studied for breast cancer association in the Eastern Finnish population (KBCP) are listed in Table 7.

Table 7.

Table 7. Genes studied for association with breast cancer risk in the Eastern Finnish (KBCP) population.

Gene	Polymorphism	Association	Significant association when stratified	OR (95% CI)	Reference
Oestrogen biosynthesis					
CYP17	5'UTR T1931C	No overall association		0.92 (0.69-1.22) for C allele containing genotypes	Mitrunen et al. 2000
Oestrogen metabolism					
CYP1A1	Ile462Val	No overall association		0.76 (0.50-1.14) for Ile/Val and Val/Val combined	Sillanpää et al. 2007
	Thr461Asn	No overall association		1.67 (0.50-5.48) for Thr/Asn	Sillanpää et al. 2007
CYP1B1	Leu432Val	No overall association	Increased risk among smokers of 1-9 cigarettes/day	2.6 (1.07-6.46) for Leu/Val 5.1 (1.30-19.89) for Val/val	Sillanpää et al. 2007
COMT	Val108/158Met	No overall association		0.81 (0.58-1.13) for Val/met and Met/Met combined	Mitrunen et al. 2001c
SULT1A1	Arg213His	No overall association	Decreased risk in premenopausal women with at least 3 pregnancies and one His allele	0.23 (CI=0.09-0.63)	Sillanpää et al. 2005b
GSTM1	Allele deletion	No overall association	Increased risk among postmenopausal GSTM1 null genotypes	1.49 (1.03-2.15)	Mitrunen et al. 2001a
GSTM3	3 bp deletion in intron 6	No overall association		1.00 (0.73-1.37) for variant allele containing genotypes	Mitrunen et al. 2001a
GSTP1	Ile105Val	No overall association		0.88 (0.66-1.18) for Ile/Val 0.57 (0.31-1.04) for Val/Val	Mitrunen et al. 2001a
GSTT1	Allele deletion	No overall association		1.18 (0.8-1.76) for null genotypes	Mitrunen et al. 2001a
MnSOD	Val16Ala	Ala allele carriers at increased risk compared to Val/Val		**1.5 (1.1-2.0)**	Mitrunen et al. 2001b
Carcinogen metabolism					
NAT2	C282T T341C		Increased risk associated with slow acetylator genotypes 282T/282T, 341C/341C, 282T/341C	1.32 (1.01-1.73) Risk higher among women smoking 5< pack-years	Sillanpää et al. 2005a
ADH1B	3'UTR A>G	No overall association		0.79 (0.59-1.05) for AG 1.38 (0.71-2.69) for GG	Cox et al. 2007

Table 7. (continued)

Gene	Polymorphism	Association	Significant association when stratified	OR (95% CI)	Reference
DNA repair					
BRCA2	Asn372His	No overall association		1.04 (0.83-1.30) for Asn/His 1.07 (0.61-1.87) for His/His	Healey et al. 2000a
XRCC1	Arg280His	No overall association		1.15 (0.80-1.66) for Arg/His and His/His combined	Metsola et al. 2005
	Arg399Gln	No overall association	Increased risk among ever smokers and at least one Gln allele	2.33 (1.30-4.19) Risk higher among women smoking 5< pack-years	Metsola et al. 2005
XPD	Lys751Gln	No overall association	Increased risk among ever smokers and Gln/Gln genotype	2.52 (1.27-5.03) Risk higher among women smoking 5< pack-years	Metsola et al. 2005
ATM	Ser49Cys	No overall association			Cox et al. 2007
Hormone- and growth factor-related genes					
IGFBP3	-202C>A	No overall association		1.20 (0.89-1.62) for CA 0.91 (0.61-1.36) for AA	Cox et al. 2007
VDR	ApaI	a allele carriers at decreased risk compared to AA		**0.73 (0.54-0.98)**	Sillanpää et al. 2004
	TaqI	No overall association		0.68 (0.41-1.12) for Tt and tt combined	Sillanpää et al. 2004
TGFB1	Leu10Pro	No overall association		0.91 (0.71-1.17) for Leu/Pro 1.14 (0.68-1.91) for Pro/Pro	Dunning et al. 2003
Cell cycle control					
STK15	Phe3Ile	No overall association		1.02 (0.78-1.34) for Phe/Ile 0.61 (0.35-1.07) for Ile/Ile	Breast Cancer Association Consortium 2006
CDKN1A	Ser31Arg	No overall association		1.27 (0.81-2.00) for Ser/Arg 3.15 (0.33-30.43) for Arg/Arg	Cox et al. 2007
Other genes					
CASP8	Asp302His	No overall association		0.82 (0.57-1.16) for Asp/His 6.53 (0.34-126.92) for His/His	Cox et al. 2007
ICAM5	Val301Ile	No overall association		0.94 (0.60-1.48) for Val/Ile 1.04 (0.66-1.63) for Ile/Ile	Cox et al. 2007
NUMA1	Ala794Gly	No overall association		0.69 (0.36-1.33) for Ala/Gly and Gly/Gly combined	Cox et al. 2007

FGFR2	intron 2 G>A rs2981582	GA and AA at increased risk compared to GG	**1.36 (1.11-1.66)** per allele	Easton et al. 2007
LSP1	intron 10 T>C rs3817198	No overall association	1.03 (0.82-1.30) per allele	Easton et al. 2007
H19	C>T rs2107425	No overall association	1.03 (0.83-1.28) per allele	Easton et al. 2007
TNRC9/ LOC643714	intron 1 C>T rs8051542	CT and TT at increased risk compared to CC	**1.37 (1.11-1.67)** per allele	Easton et al. 2007
	intron 1 A>G rs12443621	AG and GG at increased risk compared to AA	**1.26 (1.03-1.55)** per allele	Easton et al. 2007
	C>T rs380362	No overall association	1.03 (0.83-1.27) per allele	Easton et al. 2007

2.4.1 Steroid hormone biosynthesis, signalling and metabolism genes

Most of the known risk factors for breast cancer relate to the increased, prolonged or life-time oestrogen exposure. Oestrogens also undergo extensive oxidative metabolism via several enzymes, yielding catechol oestrogens (CE) and hydroxyestrone, some of which are even carcinogenic. Therefore the genes encoding enzymes involved in oestrogen and other steroid hormone biosynthesis, signalling and metabolism are strong candidates for low-penetrance genetic risk factors for breast cancer, as allelic variants in these enzymes may affect the hormone levels.

2.4.1.1 Steroid hormone biosynthesis genes

Oestrogen biosynthesis involves series of enzymatic steps from cholesterol to C-19 androgens and C-18 oestrogens (Figure 3, Table 8). Table 8 shows the genes involved in steroid hormone biosynthesis and their observed association with breast cancer risk.

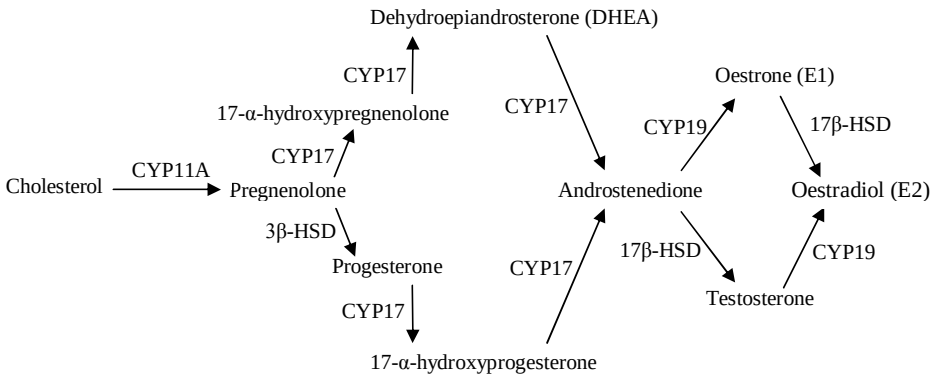


Figure 3. Oestrogen biosynthesis. CYP11A, CYP17 and CYP19 are particularly important. See Table 8 for details.

2.4.1.2 Steroid hormone signalling genes

The sex hormones bind to specific receptors and form complexes that in turn bind to sequences in the promoters of downstream, hormone-responsive genes and thus the sex hormones control the activation of responsive genes. Therefore the steroid hormone receptor genes *ESR1* (oestrogen receptor alpha) (Walter et al. 1985, Menasce et al.

Table 8. Genes involved in steroid hormone biosynthesis and signalling.

Gene, locus	Encoded Protein	Function	Studied polymorphisms	Association with breast cancer	References
Steroid hormone biosynthesis (Payne 1990, Omura and Morohashi 1995, Kristensen and Borresen-Dale 2000, Thompson and Ambrosone 2000).					
CYP11A, 15q23-q24	CYP11A	catalyses the rate-limiting step in the biosyntesis of all steroids, the cleavage of the side chain of cholesterol to form pregnenolone and progesterone			
CYP17, 10q24.3	CYP17, 17α hydroxylase/C17-20 lyase	catalyzes the rate-limiting step in ovarian and adrenal biosynthesis pathways for androstendione, immediate precursor of testosterone	5'UTR T>C	Controversial results	Feigelson et al. 1997, Dunning et al. 1998, Mitrunen et al. 2000, Einarsdottir et al. 2006
CYP19, 15q21.1	CYP19, aromatase/estrogen synthetase	aromatizes testosterone and androstendione to E2 (estradiol) and E1 (estrone)	a tetranucleotide repeat	seems to be a risk factor	Kristensen et al. 1998, Healey et al.2000b, Dunning et al. 1999a
HSD3B2, 1p13.1	3βHSD, 3β hydroxysteroid dehydrogenase	converts pregnenolone to progesterone			
EDH17B2, 16q24.1	17βHSD, 17β hydroxysteroid dehydrogenase type 2	catalyzes the interconversions of the less biologically active hormones andostenedione and estrone to their more active analogues testosterone and estradiol	several	no association	Mannermaa et al. 1994
Steroid hormone signalling					
ESR1, *ESR2*, 6q25.2, 14q	ER, oestrogen receptor alpha and oestrogen receptor beta	binds oestrogen	some	no overall effect shown	Southey et al. 1998, Cai et al. 2003, Gold et al. 2004, Zheng et al. 2003
PGR, 11q22	PR, progesterone receptor	binds progesterone	Val660Leu	haplotype may be associated with increased risk	Pooley et al. 2006, Breast Cancer Association Consortium 2006
AR, Xq11-q12	AR, androgen receptor	binds androgen	CAG repeat	controversial results	Lillie et al. 2003, Dunning et al. 1999b
SHBG, 17p13-p12	SHBG, sex hormone-binding globulin	binds the circulating sex hormones and so modulates the bioavailability of these hormones to target tissues, regulates the steroid-signalling system in target tissues	Asp327Asn	associates with increased SHBG levels and reduced risk in postmenopausal women	Dunning et al. 2004, Cui et al. 2005

1993), *ESR2* (oestrogen receptor beta) (Enmark et al. 1997), *PGR* (progesterone receptor) (Law et al. 1987, Misrahi et al. 1987) and *AR* (androgen receptor) (Chang et al. 1988, Lubahn et al. 1988), as well as *SHBG* (sex hormone-binding globulin) are candidates for breast cancer susceptibility genes. (Table 8). Women with higher circulating levels of SHBG have been detected to have a lower risk of breast cancer (Endogenous Hormones and Breast Cancer Collaborative Group 2002). Also association between higher testosterone levels and increase in breast cancer risk in postmenopausal women has been observed (Endogenous Hormones and Breast Cancer Collaborative Group 2002).

2.4.1.3 Steroid hormone metabolism genes

Oestrogens are extensively metabolised by a number of oxidative and conjugative reactions that can lead to their deactivation and subsequent elimination. Alternatively, oxidation and conjugation reactions of oestrogens may generate metabolites that have distinct biologic activities, including altered hormonal properties; genotoxicity; and/or chemotherapeutic properties. In the metabolism of oestrogen are several enzymes/genes involved (Figure 4). (Zhu and Conney 1998, Jefcoate et al. 2000, Thompson and Ambrosone 2000). (Table 9).

Oxidative metabolism of oestrogens is mediated by the same CYPs that metabolise therapeutic agents and xenobiotics. Oestrogens are metabolised via two major pathways, i.e. hydroxylation either at the A-ring or the D-ring, leading to formation of catechol oestrogens (CE) and 16α-hydroxyestrone, respectively. (Martucci and Fishman 1993, Lippert et al. 2000). Oestrogen conjugation is a major route of oestrogen metabolism. Hydroxylated oestrogens can be inactivated by *O*-methylation catalysed by COMT (Zhu and Conney 1998, Cavalieri et al. 1997). Sulfate conjugation of oestrogens and CEs is catalyzed by several members of a superfamily of cytosolic sulfotransferase enzymes, SULTs (Glatt et al. 2001). Sulfation and methylation detoxifies CEs and conjugated oestrogens are not appreciable ligands for the ERs, thus, they do not promote ER-mediated activity (Zhu and Conney 1998). CEs are the major metabolites of oestrogen, and they are intermediates for the generation of the reactive semiquinones

(CE-SQ) and guinones (CE-Q), which in turn have the ability to damage DNA and protein through redox cycling and generation of reactive oxygen species (ROS), and thus have been implicated as potential initiators of tumour formation (Cavalieri et al. 1997, Zhu and Conney 1998, Liehr 1997). Superoxide dismutases (SODs) comprise a family of metalloenzymes that catalyse the conversion of superoxide anion into hydrogen peroxide and molecular oxygen and thus protect cells from damage induced by free radicals (Winterbourn 1993). CE-Q may be conjugated with glutathione (GSH) catalysed by cytosolic glutathione S-transferases, GSTs, or reduced to CE by quinone reductase NQO1 (Cavalieri et al. 1997). (Figure 4, Table 9).

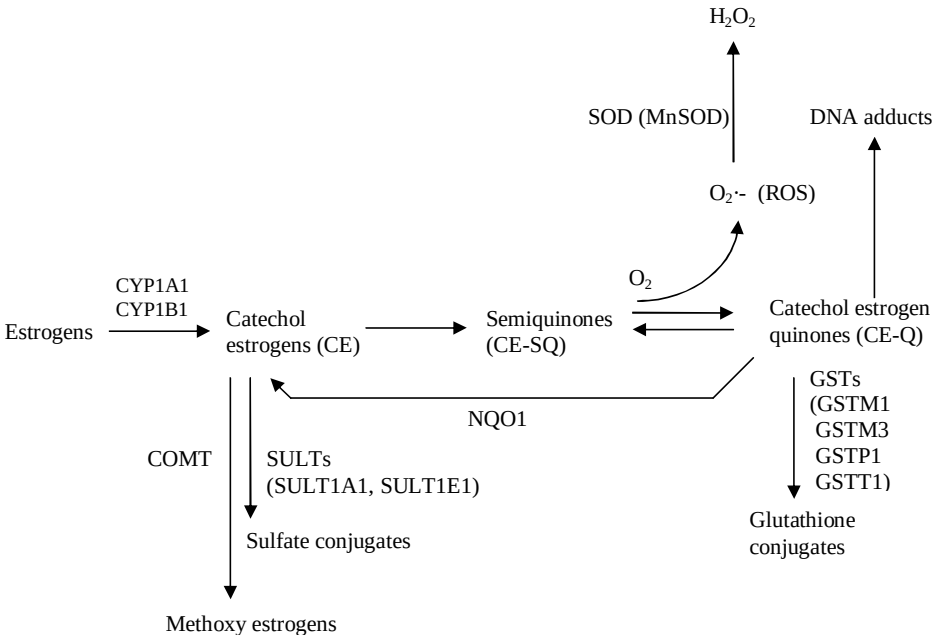


Figure 4. Oestrogen metabolism.

In addition to oestrogen metabolism, GSTs are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik 1985). GSTs catalyse the conjugation of glutathione (GSH) to a variety of endogenous and exogenous electrophiles, increasing their water solubility and excretability (Ketterer 1988). Also NQO1 is involved in metabolising numerous endogenous and

Table 9. Genes involved in steroid hormone metabolism.

Gene, locus	Encoded Protein	Function	Studied polymorphisms	Association with breast cancer	References
CYP1A1, 15q22-q24	CYP1A1, cytochrome P450 subfamily I polypeptide 1	catalyses 2-hydroxylation of oestradiol to CE, also a major role in the metabolism of several potent carcinogens e.g. in activating cigarette smoke constituents	some	association among smokers or post-menopausal women, no overall association	Ambrosone et al. 1995, Ishibe et al. 1998, Bartsch et al. 2000, Basham et al. 2001
CYP1B1, 2p22-p21	CYP1B1, cytochrome P450 subfamily I polypeptide 1	main CYP450 enzyme responsible for the 4-hydroxylation of oestradiol to CE	several	no consistent association, Leu432Val may modify risk	Sutter et al. 1994, Tang et al. 1996, Sillanpää et al. 2007
CYP1A2, 15q22-qter	CYP1A2, cytochrome P450 subfamily I polypeptide 2		some	controversial results	Le Marchand et al. 2005, Long et al. 2006
COMT 22q11.2	COMT, catechol-*O*-methyltransferase	conjugation and inactivation of catechol oestrogens by transfer of a methyl group	Val>Met change associating with decreased activity	no overall association	Thompson et al. 1998, Huang et al. 1999, Gaudet et al. 2006
SULT1A1, 16p12.1	SULT1A1, cytosolic sulfotransferase 1A1	catalyses sulfate conjugation of oestrogens and CEs, also involved in detoxification of xenobiotic compounds and in the metabolism and activation of pro-carcinogens from e.g. tobacco smoke	Arg213His, associates with decreased enzyme activity	may modify risk, no overall association	Seth et al. 2000, Zheng et al. 2001, Sillanpää et al. 2005b
SULT1E1, 4q13.1	SULT1E1, cytosolic sulfotransferase 1E1	catalyses sulfate conjugation of oestrogens and CEs, exhibits the highest affinity for oestrogens among SULTs	one study, 959G>A	decreased risk among Koreans	Choi et al. 2005
GSTM1, 1p31	GSTM1, mu-class glutathione S-transferase (GST) 1	catalyses glutathione (GSH) conjugation of catechol oestrogen quinones (CE-Q)	gene deletion	null allele associated with increased risk among postmenopausal women	Helzlsouer et al. 1998, Dunning et al. 1999a, Mitrunen et al. 2001a
GSTM3, 1p13	GSTM3, mu-class GST 3	catalyses GSH conjugation of CE-Q	3bp del in intron 6, only one study	no overall association	Mitrunen et al. 2001a
GSTP1, 11q13	GSTP1, pi family GST 1	catalyses GSH conjugation of CE-Q, predominant GST in the breast tissue and is expressed consistently in both normal and tumour breast tissue	Ile105Val, Ala114Val	overall risk not conclusive	Dunning et al. 1999a, Vogl et al. 2004, Egan et al. 2004, Mitrunen et al. 2001a
GSTT1, 22q11	GSTT1, theta family GST 1	catalyses GSH conjugation of CE-Q	gene deletion	no overall association	Dunning et al. 1999a, Vogl et al. 2004, Egan et al. 2004, Mitrunen et al. 2001a

SOD2, 6q25	MnSOD, mitochondrial manganese superoxide dismutase	catalyses the conversion of superoxide anion into hydrogen peroxide and molecular oxygen,thus protects cells from damage induced by free radicals, proposed to be a tumour suppressor	Val16Ala	overall association not conclusive	Ambrosone et al. 1999, Mitrunen et al. 2001b, Bergman et al. 2005, Egan et al. 2003, Tamimi et al. 2004b, Cai et al. 2004, Cebrian et al. 2006, Breast Cancer Association Consortium 2006, Cox et al. 2007
NQO1, 16q22.1	NQO1, NAD(P)H: quinone oxidoreductase	reduces reactive CE-Q back to hydroxy-CE, plays an important role in protecting tissues against the mutagenic, carcinogenic and cytotoxic effects, activates e.g. environmetal procarcinogens present in tobacco smoke	Pro187Ser, leading to nonfunctional enzyme	associated with increased risk in Czech and Austrian populations	Menzel et al. 2004

Table 10. Carcinogen and xenobiotics metabolism genes studied for breast cancer association.

Gene, locus	Encoded Protein	Function	Studied polymorphisms	Association with breast cancer	References
NAT1, *NAT2*, 8p22	NAT1, N-acetyltransferase 1 NAT2, N-acetyltransferase 2	participates in the metabolism of numerous pharmaceutical drugs and carcinogens (heterocyclic amines) found in tobacco smoke and diet	slow/intermediate/ fast acetylator status vs. breast cancer risk	no overall association, may modify risk	Ambrosone et al. 1996, Alberg et al. 2004, Zheng et al. 1999, Hunter et al. 1997, Millikan et al. 1998, Dunning et al. 1999a, Lee et al. 2003, Sillanpää et al. 2005a
MPO, 17q23	MPO, Myeloperoxidase	antimicrobial enzyme catalyzing hydrogen peroxide-dependent oxidation of chloride to generate hypochlorous acid, transforms xenobiotics, such as PAHs and aromatic amines to highly reactive intermediates	-463G>A, associates with reduced expression	reduced risk observed	Ahn et al. 2004, Lin et al. 2005
ADH1B, *ADH1C*, 4q22	ADH1B, alcohol dehydrogenase 1B ADH1C, alcohol dehydrogenase 1C	ADH plays a rate-limiting role in the metabolic pathway for most human ethanol oxidation	3'UTR A>G in *ADH1B* Ile350Val in *ADH1C*	no overall association	Freudenheim et al. 1999, Hines et al. 2000, Terry et al. 2006, Breast Cancer Association Consortium 2006, Cox et al. 2007

environmental quinones (Ernster and Navazio 1958, Grzeschik 1980, Povey et al. 1980, Jaiswal et al. 1988, Chen et al. 1991). It is a 2-electron reductase that detoxifies quinones derived from the oxidation of phenolic metabolites of benzene (Ernster 1967, Lind et al. 1982). In the metabolism of xenobiotics NQO1 may act as either a detoxification or activation enzyme, depending on the substrate.

2.4.2 Carcinogen and xenobiotics metabolism

Several enzymes are involved in the detoxification of xenobiotic compounds found in e.g. tobacco smoke and cooked meat. Some of these enzymes are involved in the steroid hormone metabolism also (introduced above, Table 9). The detoxifying enzymes convert carcinogenic compounds in more soluble and more readily excreted form and therefore reduce cancer risk. Some of the more soluble compounds, however, are even more carcinogenic. Thus, genetic changes that increase or decrease the activity of the detoxifying enzymes may increase the amount of reactive carcinogens and increase the risk of cancer. Such enzymes are candidates for breast cancer susceptibility and objects for genetic case-control association studies.

NAT1 and *NAT2* genes are highly polymorphic and several different allelic variants exist that determine the NAT1/NAT2-related biotransformation capacity resulting in slow, intermediate or fast/rapid acetylator phenotype (Evans et al. 1960, Deguchi et al. 1990, Vatsis et al. 1991, Blum et al. 1991, Hickman et al. 1992, Lin et al. 1993, Deguchi 1992, Butcher et al. 1998, Hughes et al. 1998). A difference in the acetylation rate can alter the proportion of specific metabolites that are formed and genotoxicity by substrates of NAT can be affected by the amount of acetylation. Relationship between acetylator phenotype and DNA damage by chemicals that undergo N-acetylation has been found and the acetylation polymorphism may be a factor in susceptibility to toxicity and perhaps carcinogenicity of these chemicals (McQueen et al. 1982). The association of NAT1 and NAT2 acetylator phenotypes and breast cancer has been studied but the findings have been very inconsistent. (Table 10).

MPO generates ROS endogenously and is linked to cancers through DNA damage and the production of carcinogens (Klebanoff 1980, Van Rensburg et al. 1992, Petruska

et al. 1992). MPO is present in breast secretions and is thought to be involved in the chemical/metabolic induction of breast cancer (Josephy 1996, Williams et al. 2000). Also, there are indications that steroid hormones regulate *MPO* expression (Bekesi et al. 2001). Thus, it is plausible to hypothesize that MPO could play a role in breast carcinogenesis, and that factors that would increase lifetime exposures to estrogens, such as age at menarche, could modify this relationship (Ahn et al. 2004). (Table 10).

Alcohol consumption may increase the risk of breast cancer by increasing the exposure to carcinogenic metabolites (Feron et al. 1991) or by influencing the levels of reproductive steroid hormones that play a critical role in breast carcinogenesis (Ginsburg et al. 1995). Alcohol dehydrogenases (ADHs) are enzymes involved in the metabolism of ethanol to acetaldehyde. ADH plays a rate-limiting role in the metabolic pathway for most human ethanol oxidation. (Bosron and Li 1986). Polymorphisms in ADH genes have been studied for breast cancer association (Table 10).

2.4.3 Other hormone- and growth factor-related genes

Breast cancer risk is known to be strongly related to endogenous hormone exposure and the genes responsive to such hormones are plausible candidates for being susceptibility genes. Also genes encoding for many growth factors involved in cellular growth, cell proliferation and differentiation, and angiogenesis are potent candidates for breast cancer risk associated factors, as all these pathways are affected in tumour growth. Several of these genes have been studied and some of them have shown association with breast cancer risk.

2.4.3.1 Insulin-like growth factor family

Insulin-like growth factor family includes the insulin-like growth factors (IGFs) I and II (IGF-I, IGF-II), six different IGF binding proteins (IGFBPs), and the receptors IGF-IR and IGF-IIR. In addition, several IGFBP proteases hydrolyze IGFBPs, resulting in release of the bound IGFs, and therefore indirectly regulate the action of IGFs. IGF family members interact with several molecules that are involved in cancer

development and progression, including the sex steroid hormones, tumour suppressors, and other growth factors. (Yu and Rohan 2000).

The IGF axis has been shown to play a role in mammary carcinogenesis (Hadsell and Bonnette 2000). IGF-I and IGFBP3 concentrations vary greatly between individuals, part of which is attributable to genetic effects (Juul et al. 1994, Juul et al. 1995, Harrela et al. 1996), and might affect the distribution of cancer risk in a population. High serum levels of IGF-I have been shown to be associated with breast cancer risk among premenopausal women (Hankinson et al. 1998, Renehan et al. 2004). The results on IGFBP3 levels and association with breast cancer risk are uncertain, possibly due to methodological reasons. A meta-analysis suggests an increase in risk with higher IGFBP3 levels (Renehan et al. 2004). However, the biological role of IGFBP3 supports the observation that higher circulating levels are protective (Al-Zahrani et al. 2006). Polymorphisms in both genes associate with the circulating levels of their respective proteins (Jernström et al. 2001, Schernhammer et al. 2003, Canzian et al. 2006, Al-Zahrani et al. 2006). (Table 11).

Growth hormone (GH) is the main stimulator of IGF-I and IGFBP3 production and genetic variants affecting the release or biological action of GH might have an effect on cancer risk. (Table 11).

2.4.3.2 Vitamin D receptor, *VDR*

Vitamin D receptor, VDR, is a ligand-activated transcription factor interacting with many pathways, including p21, fibronectin and retinoic signaling, and its actions are involved in e.g. calcium metabolism, cell proliferation and differentation, and immune function (Uitterlinden et al. 2004). Several polymorphisms (TaqI, ApaI, BsmI and poly-A which are all in LD with each other, and the only known protein polymorphism in the *VDR* gene, FokI) have been studied in the vitamin D receptor *VDR* gene but conclusive evidence on the association with increased risk of breast cancer has not been shown (Arai et al. 1997, Dunning et al. 1999b, Colin et al. 2000, Whitfield et al. 2001, de Jong et al. 2002, Uitterlinden et al. 2004, Chen et al. 2005). (Table 11).

Table 11. Other hormone- and growth factor-related genes studied for breast cancer association.

Gene, locus	Encoded Protein	Function	Studied polymorphisms	Association with breast cancer	References
IGF-I, 12q22-q24	IGF-I, insulin-like growth factor I	important role in regulating cell proliferation, differentiation, apoptosis and transformation, has characteristics of a circulating hormone and a tissue growth factor	variants that associate with raised circulating IGF-I levels	increased risk	Wen et al. 2005, Al-Zahrani et al. 2006
IGF-II, 11p15	IGF-II, insulin-like growth factor II	important role in regulating cell proliferation, differentiation, apoptosis and transformation			
IGFBP3, 7p12-p14	IGFBP3, insulin-like growth factor binding protein 3	binds IGF-I and IGF-II in the circulation, regulates the biological activity of IGFs and thus might protect against cancer development, may have IGF-independent functions, may act as a growth stimulator	variants that associate with lower circulating IGFBP3 levels, -202C>A	decreased risk	Al-Zahrani et al. 2006, Breast Cancer Association Consortium 2006, Cox et al. 2007
GH1, 17q23.3	GH, growth hormone	main stimulator of IGF-I and IGFBP3 production			
GHRH, 20q11.23	GH releasing hormone	stimulates GH release			
GHRHR, 7p15-p14	GHRHR, GH releasing hormone receptor		-261C>T	decreased risk	Wagner et al. 2006a
GHSR, 3q26-31	GH secretagogue receptor				
GHRL, 3p26-2p5	GH secretagogue receptor ligand	stimulates GH release			
SST, 3q28	SST, somatostatin	inhibits GH release	several	controversial results	Canzian et al. 2005, Wagner et al. 2006a
SSTR1-5, 14q13, 17q24, 22q13.1, 20p11.2, 16p13.3	SSTR1-5, somatostatin receptors 1-5	enhance or inhibit the synthesis and release of GH, and a pituitary-specific transcription factor, POU1F1	promoter polymorphisms in *SSTR2*	controversial results	Canzian et al. 2005, Wagner et al. 2006a
VDR, 12q11	VDR, Vitamin D receptor	ligand-activated transcription factor interacting with many pathways	TaqI, ApaI, BsmI, poly-A FokI	not associated increased risk	Dunning et al. 1999b, de Jong et al. 2002 Chen et al. 2005
VEGF, 6p21	VEGF, vascular endothelial growth factor	regulates angiogenesis, mediates vascular permeability	several	controversial results	Krippl et al. 2003, Jin et al. 2005, Kataoka et al. 2006, Jacobs et al. 2006
TGFB1, 19q13	TGFβ1, transforming growth factor β	roles in regulation of cell growth, differentiation and migration, in e.g. mammary gland development and function	Leu10Pro	increased risk	Cox et al. 2007

2.4.3.3 Vascular endothelial growth factor, *VEGF*

Formation of new blood vessels is needed for tumour growth, invasiveness and metastasis, and vascular endothelial growth factor (VEGF) is the most important regulator of angiogenesis (Ferrara et al. 1997, Ferrara et al. 2003). VEGF is an endothelial cell-specific mitogen, an angiogenic inducer and a mediator of vascular permeability (Ferrara et al. 1997). In breast cancer tissue the mRNA expression of *VEGF* is increased compared to adjacent normal tissue (Yoshiji et al. 1996). Polymorphisms in the *VEGF* have been suggested to be associated with an increased and decreased risk of breast cancer. (Table 11).

2.4.3.4 Transforming growth factor beta, *TGFB1*

TGFβ is a polypeptide cytokine that has a role in regulating cell growth, differentiation and migration. It regulates e.g. normal mammary gland development and function by activating the TGFβ signalling pathway. There is a dual-role of action in which the TGFβ signalling suppresses tumour initiation but can also promote tumour progression and metastasis when antiproliferative effect of the TGFβ signaling pathway has been overridden by other oncogenic mutations. (Bierie and Moses 2006, Derynck et al. 2001).

A Leu10Pro polymorphism resides in the signal peptide sequence of TGFβ1 and the peptide with Pro at residue 10 causes a 2.8-fold increase in secretion compared with the Leu form (Dunning et al. 2003). In a large collaborative study some evidence for association with *TGFB1* Leu10Pro and breast cancer risk was shown (Breast Cancer Association Consortium 2006). This was further confirmed in an even larger sample material (Cox et al. 2007). The analysis of Leu10Pro variant showed a significant dose-dependent association of the Pro allele with increased risk of invasive breast cancer (per-allele OR 1.08, CI=1.04-1.11, P(trend)=2.8 x 10^{-5}), and it was estimated to account for approximately 0.2 % of the excess familial risk of breast cancer in populations of European ancestry (Cox et al. 2007). (Table 11).

2.4.4 Genes involved in double-stranded DNA break repair

Double-strand breaks are frequently induced by e.g. certain chemicals, ionizing radiation, and mechanical stress, and they are particularly difficult to repair compared with other types of DNA damage. Inability to correctly repair DSBs can cause chromosomal loss, translocations and deletions that may lead to activation of proto-oncogenes, loss of function of tumour suppressor genes or global genomic instability and thus, lead to tumorigenesis. (Khanna and Jackson 2001). The genes which normally participate in the error-free repair of breaks in double-stranded DNA predispose to breast and other cancers when inactivated and common variants of DSB-involved genes have been implicated in epidemiological studies of breast cancer risk.

Two general forms of DSB repair are found within eukaryotic cells: non-homologous end-joining (NHEJ) and homologous recombination (HR). Several types of homologous repair exist; gene conversion, break-induced replication (BIR) and single-strand annealing (SSA). Similarly, there are several alternative end-joining mechanisms. (Haber 2000). NHEJ repairs broken DNA ends by joining the broken strands together and it does not need an undamaged partner and does not rely on extensive homologies between the two recombining ends. Consequently, NHEJ is often prone to error, and small deletions often occur at the site of fusion. HR uses homologous chromosomal or sister chromatid DNA as template for synthesis of new error-free DNA. In HR, the DNA ends are first resected in the 5' to 3' direction by nucleases. The resulting 3' single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner. Following branch migration, the resulting DNA crossovers are resolved to yield two intact DNA molecules. In contrast to NHEJ the HR is a high fidelity process. (Khanna and Jackson 2001).

In humans, several genes are involved in the NHEJ (*KU70*, *XRCC5*, *DNA-PK*, *XRCC4*, *LIG4*) and HR (*ATM*, *BRCA1*, *BRCA2*, *RAD51*, *RAD52*, *RAD54*, *RAD51B*, *RAD51C*, *RAD51D*, *RPA*, *XRCC2*, *XRCC3*, *DMC1*, *ZNF350=ZBRK1*, *BRIP1=BACH1*) and proteins encoded by some of them (*NBS1*, *MRE11*, *RAD50*) have functions in both pathways (van Gent et al. 2001). Polymorphisms have been studied in several of these genes and some studies have reported associations, but others not (Table 12).

Table 12. Genes involved in double-stranded DNA break repair that have been studied for breast cancer association.

Gene, locus	Contribution to breast cancer risk	References
XRCC2, 7q36.1	suggested to modify risk, significant main effect not confirmed	Kuschel et al. 2002, Rafii et al. 2002, Breast Cancer Association Consortium 2006
LIG4, 13q22-q34	suggested to modify risk, significant main effect not confirmed	Kuschel et al. 2002, Breast Cancer Association Consortium 2006
XRCC3, 14q32.3	suggested association with weak risk, significant main effect not confirmed	Kuschel et al. 2002, Garcia-Closas et al. 2006, Breast Cancer Association Consortium 2006
XRCC4, *Ku70*, 5q13-q14	suggested association among Taiwanese	Fu et al. 2003
RAD52, 12p13-p12.2	suggested association among Koreans	Lee et al. 2005b
RAD51, 15q15.1	modifies risk among *BRCA* mutation carriers, no role in sporadic breast cancer	Levy-Lahad et al. 2001, Wang et al. 2001a
RAD51D, 17q11	suggested association with increased risk	Rodriguez-Lopez et al. 2004
ZNF350	suggested association in familial high-risk subgroup	Garcia-Glosas et al. 2006
BRIP1 (*BACH1*), 17q22	truncating mutations are low-penetrance breast cancer susceptibility alleles in familial breast cancer, estimated breast cancer-attributed fraction of 0.20 %, SNPs not associated	Sigurdson et al. 2004, Seal et al. 2006, Song et al. 2007
FANCD2, 3p25.3	suggested association with increased risk	Barroso et al. 2006
NBS1, 8q21	suggested association with increased risk, mutations seem to contribute to risk	Kuschel et al. 2002, Lu et al. 2006, Steffen et al. 2006
RAD50, 5q31	suggested low-penetrance risk allele among Northern Finnish breast cancer cases	Heikkinen et al. 2006
BARD1, 2q34	see text for details	

2.4.4.1 *BARD1*

The BRCA1-associated ring domain 1 (BARD1) protein was discovered as a binding partner of BRCA1 (Wu et al. 1996). BARD1 and BRCA1 have several features in common: similar protein structure, the embryonic lethality of their respective knockout mice and induction of genetic instability when depleted from cells (Irminger-Finger and Jefford 2006). BARD1 and BRCA1 form a functional heterodimer through the binding of their RING-finger domains (Wu et al. 1996, Meza et al. 1998). The interaction is required for several of the cellular and tumour suppressor functions of BRCA1. BRCA1-BARD1 heterodimer has been described as the functional unit in DSB repair (Jasin 2002). BARD1 and BRCA1 also associate with other repair proteins, such as the MSH2-MSH6 complex, and this interaction indicates a role in DNA mismatch repair (Wang et al. 2001b). Several reports have also demonstrated BRCA1-independent functions of BARD1, primarily in apoptosis, and BRCA1-independent increased

expression of BARD1 during mitosis, indicating that BARD1 on its own might have crucial functions. Its cancer-associated activities seem to centre on two major pathways: as an essential component of a ubiquitin ligase involved in DNA repair and cell-cycle regulation, and p53-mediated apoptosis. (Irminger-Finger and Jefford 2006).

BARD1 gene locates on chromosome 2q34 (Wu et al. 1996, Thai et al. 1998). Germline *BARD1* mutations have been found in few cases of sporadic and familial, non-*BRCA1/2*, breast and ovarian cancer patients and some tumours, but they account for only a small fraction of cases of familial breast cancer overall (Thai et al. 1998, Ghimenti et al. 2002, Ishitobi et al. 2003, Karppinen et al. 2004, Sauer and Andrulis 2005). One missense alteration, Cys557Ser has been suggested to associate with a slightly increased risk of familial breast cancer (Ghimenti et al. 2002, Karppinen et al. 2006). Increase in risk was also observed in Icelandic case-control study, and the risk among *BRCA2* 999del5 mutation carriers was further increased if they also carried the *BARD1* 557Ser (Stacey et al. 2006). In the Chinese population other *BARD1* variant alleles have been suggested to be protective (Huo et al. 2006).

2.4.5 Genes involved in single-stranded DNA damage repair mechanisms

2.4.5.1 Base-excision repair

Base-excision repair (BER) is an important DNA repair pathway responsible for the repair of single-stranded base damage resulting from X-rays, oxygen radicals, methylation, deamination, and hydroxylation. BER acts on small lesions and involves release of the damaged base and removal of up to a few neighboring nucleotides. BER is particularly relevant for preventing mutagenesis. BER pathway includes several proteins and the genes encoding them have numerous genetic variants. These genes include *XRCC1*, *ADPRT* (PARP-1), *PCNA*, *FEN1*, *OGG1*, *MUTYH*, *APEX1=APE1*, *LIG3*, *LIG1*, *NTH1*, and *POLB* (DNA polymerase β). XRCC1 protein interacts with DNA polymerase β, DNA ligase III, PARP-1, and APE1 in the final ligation stage of the BER DNA repair pathway, and of the BER genes *XRCC1* is the most studied for breast cancer association. (Hoeijimakers 2001, Goode et al. 2002b). (Table 13).

Table 13. Polymorphisms studied for breast cancer association in genes involved in base-excision repair (BER) and nucleotide-excision repair (NER).

Gene, locus	Polymorphism	Contribution to breast cancer	References
BER			
XRCC1, 19q13.2	Arg399Gln	suggested association among African-Americans and Asian populations, suggested to modify risk among smokers or with family history, no overall association confirmed	Duell et al. 2001, Kim et al. 2002, Figueiredo et al. 2004 ,Metsola et al. 2005, Chacko et al. 2005, Zhang et al. 2006, Breast Cancer Association Consortium 2006
NER			
XPD (*ERCC2*), 19q13.2	Asp312Asn	association observed in some but not confirmed in larger studies	Justenhoven et al. 2004, Försti et al. 2004, Kuschel et al. 2005, Breast Cancer Association Consortium 2006
	Lys751Gln	suggested to associate with risk and modify risk among smokers (Finns)	Justenhoven et al. 2004, Terry et al. 2004, Metsola et al. 2005
	Asp1140His	marginal association among Finns	Kumar et al. 2003
ERCC1,19q13.2	8092A>C	suggested increased risk among Koreans	Lee et al. 2005b
ERCC4 (*XPF*), 16p13.3	intronic variant	suggested protective	Milne et al. 2006

2.4.5.2 Nucleotide-excision repair

Nucleotide-excision repair (NER) deals with the wide class of bulky helix-distorting lesions that interfere with base pairing and generally obstruct transcription and normal replication. Most NER lesions arise from exogenous sources and affect only one of the DNA strands (single-stranded). Two NER subpathways exist: global genome NER (GG-NER) and transcription-coupled repair (TCR). (Hoeijimakers 2001). Numerous proteins are involved in NER and genes encoding them include *XPA-XPG*, *ERCC1*, *RAD23A*, *RAD23B*, *CETN2*, *RPA1-3*, *GTF2H1-4*, *DDB1*, *DDB2*, *CSA*, *CSB*, *XAB2*, *BRCA1*, and *BRCA2* (Friedberg 2001). Some of these genes are candidates and have been studied for breast cancer risk association. *XPD* (=*ERCC2*) encodes a protein that is an integral member of the basal transcription factor BTF2-TFIIH complex, and it is the most studied of the NER genes for breast cancer association. (Table 13).

2.4.5.3 Mismatch repair

Mismatch repair (MMR) removes nucleotides mispaired by DNA polymerases and insertion/deletion loops (ranging from one to ten or more bases) that result from slippage during replication of repetitive sequences or during recombination. Defects in this system dramatically increase mutation rates, fuelling the process of oncogenesis.

Genes involved in MMR are *hMLH1*, *hMSH2*, *hMSH3*, *hMSH6*, *hPMS1* and *hPMS2*. (Hoeijimakers 2001). So far polymorphism only in *hMLH1* has been associated with increased risk of breast cancer in Korean population (Lee et al. 2005b).

2.4.6 Genes involved in cell cycle control

Cell cycle control is critical for normal growth and differentiation. Defects in checkpoints contribute to genetic instability and cancer. Genes encoding proteins that are involved in controlling cell cycle progression, include e.g. *TP53*, *CHEK2*, *ATM*, *RB1*, *CDKN1A*, *CDKN1B*, *CDKN2A*, *CCND1* and *STK15*.

The *STK15* (*AURORA2*/*AURKA*/*BTAK*/*ARK1*) gene on chromosome 20q13 encodes a serine/threonine kinase that acts as a key regulator of mitotic chromosome segregation, and has a role in the etiology of cancer (Shindo et al. 1998, Hirota et al. 2003, Meraldi et al. 2004). A Phe31Ile polymorphism in *STK15* has been reported to be a low-penetrance susceptibility allele affecting multiple cancer types (Ewart-Toland et al. 2005) and it has been studied for breast cancer association but the results are inconsistent. (Table 14).

Table 14. Breast cancer association of genes involved in cell cycle control.

Gene, locus	Variation	Contribution to breast cancer	References
STK15, 20q13	Phe31Ile	differing results, suggested increased risk in women < 40 years of age	Sun et al. 2004, Ewart-Toland et al. 2005, Cox et al. 2006, Fletcher et al. 2006, Breast Cancer Association Consortium 2006
CDKN2A, 9p21	inherited mutations	predispose to breast cancer in the context of a syndrome of melanoma, pancreatic cancer, and breast cancer in Swedish families	Borg et al. 2000
	Ala148Thr	suggested association in Polish population	Debniak et al. 2005

CDKN2A gene on chromosome 9p21 is a tumour suppressor gene that is involved in susceptibility of malignant melanoma and familial pancreatic carcinoma (Kamb et al. 1994, Monzon et al. 1998, Whelan et al. 1995, Sherr 2001). The p16 protein encoded by *CDKN2A* is a cyclin dependent kinase inhibitor that suppresses cell proliferation (Serrano et al. 1993). p16 is a cell-cycle regulator that inhibits the activities of cdk4 and cdk6, two protein kinases that in turn phosphorylate the retinoblastoma protein which is

necessary for progression through the G1 cell cycle checkpoint (Serrano et al. 1993, Sherr 2001). (Table 14).

2.4.7 Proto-oncogenes

Proto-oncogenes are involved in the regulation of normal cell growth and differentiation. Mutations in proto-oncogenes lead to disturbances in the cell cycle and can result in abnormal growth of proliferation. Germ-line mutations in proto-oncogenes have not been found in syndromes conferring an increased susceptibility to breast cancer. However, polymorphisms have been examined which would give a modest elevation in risk. (Weber and Nathanson 2000).

The proto-oncogene *HRAS1* locates on chromosome 11p15 and encodes a protein involved in mitogenic signalling, the process by which an extracellular growth factor signal is transmitted to the nucleus (Junien et al. 1984, Barbacid 1987). A minisatellite composed of 30-100 units of a 28-base pair repeat is tightly linked to *HRAS1* (Capon et al. 1983). The carriers of a certain class of rare alleles of the minisatellite locus are proposed to be at increased risk of breast cancer, the relative risk being approximately twofold (Krontiris et al. 1993). However, two most recent studies using a more accurate method for allele sizing found no overall association and it seems that the earlier results are false due to misclassification of alleles resulting from methodological reasons (Firgaira et al. 1999, Tamimi et al. 2003). These findings suggested that the putative associations of *HRAS1* minisatellite alleles and breast cancer need to be re-evaluated with the new method of sizing alleles.

In one study an increased risk of breast cancer was observed to be associated with an Asn11Ser polymorphism of the *c-MYC* gene (Wirtenberger et al. 2005). *c-MYC* encodes for a transcription factor that is involved in cell proliferation and differentiation, cell cycle progression, malignant transformation, genomic instability/DNA-damage sensitization, and apoptosis (Patel et al. 2004).

HER2 (*ERBB2*, *NEU*) proto-oncogene gene locates on chromosome 17q21 and encodes for a transmembrane glycoprotein p185 with a tyrosine kinase activity (Schechter et al. 1984, Yang-Feng et al. 1985, Coussens et al. 1985, Akiyama et al.

1986). p185 is a member of the epidermal growth factor receptor family that controls a variety of cellular functions, including cell division, migration, adhesion, differentiation, and apoptosis (Yarden and Sliwkowski 2001). *HER2* amplification or overexpression is seen in about 25 % of breast cancers and has been associated with metastatic phenotype, endocrine therapy unresponsiveness, and poor prognosis (Menard et al. 2004). A common variant in *HER2*, a SNP Ile655Val in the transmembrane region could result in increased protein tyrosine kinase activity (Papewalis et al. 1991, Fleishman et al. 2002). Several studies have evaluated the association of this *HER2* polymorphism with breast cancer risk. Xie et al. (2000) first reported an increased risk of breast cancer among Val allele carriers in Chinese population. Several larger studies found no association (Millikan et al. 2003, Han et al. 2005, Benusiglio et al. 2005) but others suggested even a protective effect (Nelson et al. 2005, Cox et al. 2005c), leaving the question of *HER2* and genetic breast cancer association still controversial.

2.4.8 Other genes studied for breast cancer association

Several other genes from different pathways have been studied for association with breast cancer and many of them have not shown any significant associations. Genes with significant association with breast cancer risk are introduced here.

The *CASP8* gene on chromosome 2q33 encodes caspase-8, one of the initiator caspases that transduce apoptotic signals from the death receptors at the cell surface (Kischkel et al. 1998, Hengartner 2000). Caspase-8 is activated by external death signals and in response to DNA damage (Hengartner 2000). *CASP8* Asp302His has been estimated to account for approximately 0.3 % of the excess familial risk of breast cancer in populations of European ancestry, and this site was not polymorphic in Korean, Han Chinese or Japanese women. The functional consequences of the Asp to His substitution are not yet known, and further experiments are needed to establish whether Asp302His itself, or another variant in LD with it, is causative. (Cox et al. 2007). (Table 15).

AIB1 (*NCOA3*) gene locates on chromosome 20q11 and encodes the AIB1/SRC-3 (amplified in breast cancer 1/steroid receptor coactivator 3) protein (Anzick et al. 1997).

AIB1 is amplified and overexpressed in breast and ovarian tumours and it enhances ER-dependent transcription (Anzick et al. 1997). (Table 15).

Table 15. Other genes associating with breast cancer risk.

Gene, locus	Polymorphism	Contribution to breast cancer	References
CASP8, 2q33	Asp302His	reduced risk allele dose-dependent, per allele OR 0.88, CI=0.84-0.92, $P_{trend}=1.1x10^{-7}$	MacPherson et al. 2004, Frank et al. 2005, Breast Cancer Association Consortium 2006, Cox et al. 2007
AIB1 (*NCOA3*), 20q11	3'UTR CAG/CAA repeat length polymorphism	proposed to modify risk among *BRCA* mutation carriers but not confirmed in all studies	Rebbeck et al. 2001, Kadouri et al. 2004, Haiman et al. 2000, Wilkening et al. 2005, Hughes et al. 2005, Spurdle et al. 2006
	Gln586His Thr960Thr	suggested protective effect among *BRCA* mutation negative	Burwinkel et al. 2005
TNFα, 6p21.3	-417G>A	increased risk suggested	Gaudet et al. 2007
	-308	conflicting results	de Jong et al. 2003
TNFβ, 6p21.3	A252G	suggested association in Korean population	Lee et al. 2005c
FGFR2, 10q26	intron 2 C>T (rs2981582)	increased risk allele dose-dependent	Easton et al. 2007

The HLA class III subregion has been suggested to have a role in susceptibility to breast cancer (de Jong et al. 2003). Candidate genes in this region include *TNFα* (tumour necrosis factor) and *TNFβ*. Association with breast cancer risk and polymorphisms in these genes have not been confirmed. (Table 15).

Recently, two large genome-wide searches identified the gene encoding fibroblast growth factor receptor 2 (*FGFR2*) on chromosome 10q26 to associate with increased risk of breast cancer (Easton et al. 2007, Hunter et al. 2007). FGFR2 is a receptor tyrosine kinase that is amplified and overexpressed in 5-10 % of breast tumours (Moffa et al. 2004, Adnane et al. 1991, Jang et al. 2001). Somatic missense mutations of *FGFR2* that are likely to be implicated in cancer development have also been demonstrated in primary tumours and cell lines of multiple tumour types (Jang et al. 2001, Greenman et al. 2007). The minor allele of SNP rs2981582 in *FGFR2* gene in Europeans was associated with an increased risk in a dose-dependent manner, with a higher risk in homozygous (10.5 %) than in heterozygous carriers (6.7 %) or common homozygotes (5.5 %) (Easton et al. 2007). Haplotype analysis of 7 SNPs indicated that multiple haplotypes carrying the minor allele of rs2981582 were associated with an increased risk of breast cancer, implying that the association was being driven by

rs2981582 itself or a variant strongly correlated with it. However, the variant causally related to breast cancer risk could not be identified and functional studies will be required to determine which variant it is. (Easton et al. 2007). (Table 15).

2.5 Strategies for finding new breast cancer susceptibility genes

2.5.1 Linkage disequilibrium

Linkage disequilibrium (LD) is the non-random association of alleles at adjacent loci. The loci are in LD when a particular allele at one locus is found together on the same chromosome with a specific allele at a second locus, more often than is mathematically expected based on the allele frequencies. The extent and strength of LD in a population depends on time and recombinational distance between markers. LD gradually decays as the function of the number of generations, and the time required for disappearance of LD depends on the distance between the loci (Figure 5). (Jorde 1995).

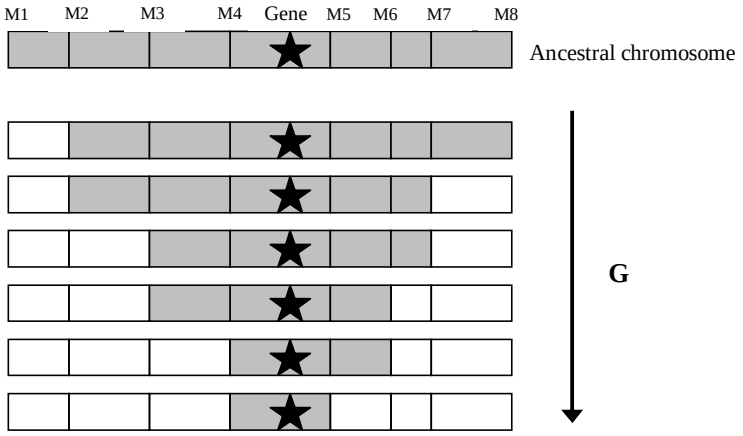


Figure 5. Linkage disequilibrium around an ancestral mutation (a black star). Recombination causes the LD to gradually decay and limits the extent of the region of association over time but markers that are physically close tend to remain associated with the ancestral mutation. Chromosomal regions derived from the ancestral chromosome are shown in grey and new regions introduced by recombination in white. G = generations during which recombination occurs. M1-M8 = marker alleles.

Power to detect LD tends to be greatest when a single disease-causing mutation that accounts for a large proportion of the phenotypic variance of a trait has arisen recently on a relatively uncommon haplotype background (Jorde 2000). Mutations responsible for complex diseases will often persist for long periods of time because they are typically less subject to the effects of natural selection than are mutations responsible for Mendelian diseases. This can further diminish LD and the resultant power to detect it (Jorde 2000). In addition to recombination and natural selection LD is also influenced by other factors complicating the relationship between LD and physical distance, e.g. mutation, chromosomal location (variable recombination rates), demographic history (population structure, admixture or migration (gene flow), population growth, genetic drift), and gene conversion (Laan and Pääbo 1997, Zavattari et al. 2000, Abecasis et al. 2001, Daly et al. 2001, Pritchard and Przeworski 2001, Ardlie et al. 2001). The strength of LD and the distance over which it extends varies from one region of the genome to another. LD also varies among populations, with European populations typically showing lower nucleotide diversity and greater LD than African populations (Reich et al. 2001). In young populations LD is thought to extend further than in older populations (Mohlke et al. 2001, Ophoff et al. 2002, Varilo et al. 2003). Also microsatellite markers have been observed to detect LD over longer distances than SNP markers (Ophoff et al. 2002, Varilo et al. 2003). LD can be measured in a number of ways but the most important pairwise measures of LD are r^2 and |D'|. Both range from 0 (no disequilibrium) to 1 (complete disequilibrium), but their interpretation is different. |D'| is a better measure for describing the patterns of LD and r^2 is of more direct relevance to the power of association studies. (Pharoah et al. 2004).

The pattern of LD is structured in stretches of consistently high LD interspersed with short intervals of rapid breakdown (Daly et al. 2001) (Figure 6). These LD blocks result from the localization of recombination to irregularly spaced hot spots (Daly et al. 2001, Jeffreys et al. 2001). In the regions of high recombination rate LD is lower and in regions of low recombination LD is higher. The common haplotypes underlying blocks of LD can be defined by testing a sufficiently large collection of SNPs. Focusing on haplotype blocks greatly clarifies LD analyses. Once the haplotype blocks are identified they can be treated as alleles and tested for LD, thereby reflecting the underlying

population variation more accurately than any individual SNP (Daly et al. 2001, Johnson et al. 2001). By determining the extended haplotypes at any given locus in a population it is possible to identify exactly which SNPs will be redundant and which will be essential to capture the haplotypes of a gene or a region of LD, i.e. the haplotype-tagging SNPs, htSNPs (Johnson et al. 2001). Haplotype maps have been constructed and information on htSNPs can be found in databases (Patil et al. 2001, Dawson et al. 2002, Gabriel et al. 2002, Phillips et al. 2003, International HapMap Consortium 2003, International HapMap Consortium 2005, Conrad et al. 2006).

Linkage disequilibrium can be utilized in identification of disease loci e.g. in initial locus positioning, restricting and fine mapping the critical chromosomal region and searching for shared haplotypes, using different strategies (de la Chapelle and Wright 1998, Kruglyak 1999, Peltonen 2000).

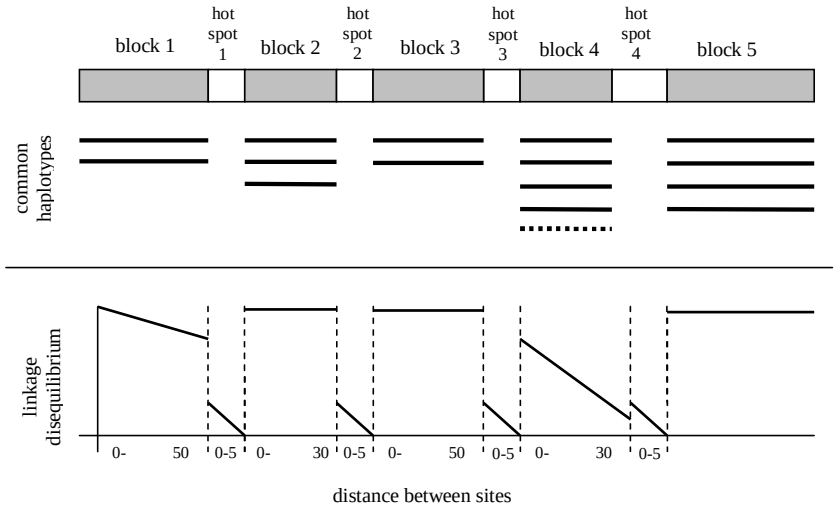


Figure 6. The block-like structure of LD, with regions of low haplotype diversity separated by recombinational hot spots. Lines below the blocks represent examples of the number of common haplotypes that might be present for such blocks. The graphs plot LD as a function of distance. The plots show that within a block LD decays gradually with distance, or not at all. Within hot-spot areas LD falls away much more rapidly with distance. (Adapted from Goldstein 2001).

2.5.2 Positional cloning of Mendelian genes

Positional cloning begins with linkage analysis. In pedigrees with a hereditary (monogenic) disease the affected members share the disease phenotype and with an increased probability they also share the genomic region that carries the disease gene. Linkage analysis identifies the chromosomal region shared by the affected members in a family. This region is estimated by genotyping genetic markers (e.g. STRs), typically spaced by 5-10 cM, and then using the marker genotypes and the data on their possibilities of transmission in the family in the linkage analysis. A calculated LOD (logarithm of odds) score indicates the probability of the chromosomal region to be the one with the disease gene. The LOD scores of studied families can be combined and so strengthen the result and improve the finding of a true linkage. In addition to family or pedigree-based linkage analysis, sib-pairs or trios can be used. After the initial locus positioning LD-based fine-mapping is needed to identify the candidate-genes. In an ideal case only a few genes in a region of 1 cM are restricted as candidates. Mutation analysis of the candidate-genes finally indicates the disease gene, mutations in which associate with the disease phenotype. (Peltonen et al. 2000, Botstein and Risch 2003). Functional studies further confirm the gene-disease association.

Family-based linkage studies have been the foundation for the many successes in mapping of genes associated with Mendelian disorders, including many rare family cancer syndromes such as multiple endocrine neoplasia type 2 and adenomatous polyposis coli (APC). Linkage studies have also been successful in mapping *BRCA1* and *BRCA2*, and genes for other common cancers, such as *MSH2* and *MLH1* in colorectal cancer and *CDKN2A* in melanoma. Success of this approach is however, limited to genes with rare, highly penetrant alleles. Linkage studies lack power to detect the alleles conferring moderate risks that are likely to be the norm in complex disease. Genetic heterogeneity limits the usefulness of combining data from multiple families, as different genes may be responsible for disease clustering in different families. Within families, phenocopies and incomplete penetrance are a problem, as the carrier status of a putative disease allele cannot be definitively inferred from disease status. (Pharoah et al. 2004).

2.5.3 Strategies for finding genes for complex diseases

2.5.3.1 Case-control association study

Although linkage studies have been highly successful in mapping the genes that underlie monogenic disorders, these studies are of limited use for investigating predisposition to polygenic disease, such as cancer (Pharoah et al. 2004). The main alternative to linkage studies for disease gene mapping is the association study, in which the frequency of a genetic variant in diseased individuals (cases) is compared to the frequency of the variant in individuals without the disease (controls) (Risch 2000, Cardon and Bell 2001). Allelic association is present when the distribution of genotypes differs in cases and controls. Linkage disequilibrium is utilized in association studies as it is based on the assumption that the affected share a genetic variant which is so close to the marker locus that the probability of a recombination event to occur between them is minimal. The observed marker-disease association in a region of high marker-marker LD in a case-control set indicates that the locus related to disease susceptibility is nearby. (Pharoah et al. 2004). Association studies for disease genes are generally based on the "common variant, common disease" hypothesis (Chakravarti 1999). Genetic variants arising a long time ago might have become common in the present population at frequencies ranging from a few percent upwards. Some of these variants might predispose to common diseases, and combinations of these variants are proposed to underlie differences in disease susceptibility. One possibility to enhance the detection of an association is haplotype analysis which combines the information of individual markers (Baynes et al. 2007). Haplotypes mark recognizable chromosomal segments that can be tracked through pedigrees and through populations when not broken up by recombination and be treated for mapping purposes as alleles. (Strachan and Read 2003). Genetic association studies provide an efficient design for identifying common variants that confer modest disease risks. (Pharoah et al. 2004). The power of association studies can be increased by selecting cases with a family history of the studied disease (Antoniou and Easton 2003).

2.5.3.1.1Genome-wide approach

The strategies of association studies are the genome-wide approach and a candidate-gene (or pathway) approach. In the genome-wide approach the markers are genotyped across the whole genome, instead of only a few candidate-genes. One starting point is to use microsatellite (STR) markers for screening the genome to find associated chromosomal regions. This application utilizes linkage disequilibrium and sets of STR markers that are traditionally used for linkage studies and spaced by 5-10 cM. Marker allele distributions are compared between cases and controls, and statistically significant differences show markers that are in LD with the disease (breast cancer). LD regions are then covered with additional markers spaced more closely, to refine the association. Especially in younger populations the linkage disequilibrium is thought to extend further and in general STR markers detect LD over longer distances than SNP markers.

At present, mostly due to technical ease, the use of SNP markers is predominant and there are two general strategies for genome-wide approach. The first uses quasi-random or anonymous SNPs that are spaced accross the genome. The second strategy uses sets of LD-based htSNPs that are specifically chosen. (Jorgenson and Witte 2006). Current understanding is that 200,000-500,000 SNPs would be needed to adequately tag all SNPs with a minor allele frequency of 5 % or more (Kruglyak 1999). It has been suggested that concentrating on SNPs on genic regions in genome-wide association studies can be more efficient for detecting causal variants than the existing indirect approaches, which attempt to capture information on all variants (Jorgenson and Witte 2006).

2.5.3.1.2Candidate-gene approach

The alternative to genome-wide association studies is the candidate-gene approach, in which the studies are focused on candidate-genes that encode proteins thought to be involved in the process of interest, e.g. in carcinogenesis, such as those involved in apoptosis, cell-cycle control, carcinogen metabolism or DNA repair. It is also possible to select the candidate-genes based on initial linkage or microsatellite marker association studies or animal models. Ideally, single nucleotide polymorphisms of

known functional significance are studied. The rationale for the candidate-gene approach is that by maximising the biological plausibility, the chances of success are increased. However, this approach is limited by its reliance on existing knowledge to identify candidate-genes based on function. (Pharoah et al. 2004).

In the ideal candidate-gene association study the common polymorphisms in the studied population and the extent of LD are first identified in order to estimate the minimum set of polymorphisms that need to be genotyped to be able to report on all other polymorphisms (htSNPs). When an association study is completed there might be residual uncertainty about the risks that are associated with the susceptibility allele. Also, the observed associated marker might not be the true functional variant and it has to be determined separately, possibly requiring functional assays. (Pharoah et al. 2004). Difficulties in assessing the actual risk-associated variant are also due to the fact that in most Western populations the linkage disequilibrium within genes is so uniform that differentiating the associated variant is impossible. At this point using another ancestral population, e.g. African or Asian, might provide further support due to weaker LD. (Risch et al. 2001, Easton et al. 2007).

Candidate-gene approach can be improved by sample selection. By choosing cases with a family history of the studied disease, e.g. breast cancer, or bilateral cases, improves the power and the sample size needed to detect an association is reduced, relative to a standard case-control association study (Antoniou and Easton 2003). The relative efficiency obtained by using familial cases is greater for rarer alleles (Antoniou and Easton 2003). Enrichment for genetic factors may also be sought by selecting cases with an early onset, although it may have a limited effect on the power to detect association in breast cancer (Antoniou and Easton 2003). Using a founder population has been extremely powerful for mapping Mendelian disorders because genetic heterogeneity is reduced and the disease might be associated with a single founder haplotype. In the search for genes for complex disorders the advantages are less clear but in the study of rarer variants of more recent origin a founder population might be useful. (Pharoah et al. 2004, Dunning et al. 2000). (Table 16).

Current efforts to identify genetic variation have concentrated on common polymorphisms. However, it is possible that much of the variation in cancer risk is due

to rarer alleles. In fact, virtually all susceptibility alleles identified so far have frequencies less than 1 %. The rarity of these variants is likely to be due to the fact that they are of recent origin and have not had time to spread through the population. It is possible that rare alleles turn out to be the more important component in cancer susceptibility. (Pharoah et al. 2004).

Table 16. Benefits of isolated versus outbred populations in genetic studies. (Adapted from Peltonen et al. 2000).

Population isolate	Outbred population
Higher prevalence for some diseases	More affected people
More inbreeding and the opportunity to map recessive genes	More opportunity for replication
More uniform genetic background	Markers more polymorphic
Good genealogical records	Genes mapped pertinent to more humanity
Easier to standardise phenotypic definitions	
Wider intervals of LD	
Closer to HWE	
Less migration and more intact families	
More uniform environment	

2.5.4 The Finnish population

Finland has been inhabited continuously at least since the last glacial period, for some 10 000 years (Norio 2003a). It is not known exactly who these original arctic people were but most likely they were not the only immigrants who arrived in Finland, thus the country was inhabited by small immigrant groups mainly from the South and East but also from the West (Peltonen et al. 1999, Norio 2003a). Both paternally inherited Y-chromosomal haplotypes and maternally inherited mitochondrial sequences show an exceptional decrease in the genetic diversity of the Finns when compared with other European populations (Sajantila et al. 1996). The establishment of agriculture in Finland may have happened about 3 500 years ago, although it is not certain at which time and by which people it was started (Norio 2003a). In the historical era, the expansion of agriculture to "uninhabited" hunting grounds has had a decisive role in the generation of the Finnish disease heritage (Norio 2003b).

The size of the Finnish population has been estimated to have been about 2 500-10 000 until 3 300 years ago. In 1100 the population is estimated to have been less than 50 000 and in 1500-1700 around 300 000. (Norio 2003a). The first inhabited areas were the

Southern, Southeast, Southwest and coastal regions, the so called early-settlement region (Figure 7). The population expansion, populating the late-settlement region, i.e. the Eastern and Northern parts of Finland, started only after 1500 from the region of South Savo (Figure 7). Small groups of founders settled in uninhabited areas resulting in formation of small, rural sub-isolates (multiple founder effects). The population of the late-settlement region, including Northern Savo, has been expanding for 20-25 generations in isolation (mainly because of distance) and is still characterized by sub-isolates with 50 000 or less inhabitants (Nevanlinna 1972, Norio et al. 1973, Norio 2003b).

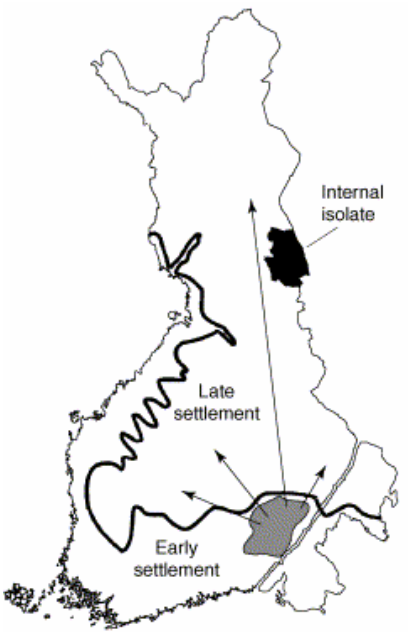


Figure 7. The early and late-settlement regions in Finland. The region of South Savo is shaded in grey. An example of a regional (internal) isolate of Kuusamo is also shown (shaded in black). (Adapted from Varilo and Peltonen 2004).

The main expansion of the Finnish population occurred in remarkable isolation due to linguistic, religious, cultural and geographical barriers. There has been very little immigration in Finland in the last 80-100 generations of expansion and population bottlenecks have occurred. Epidemics and particularly famines in a Northern country

sensitive to frost have probably been more important than wars in respect of the bottlenecks. The famine with its accompanying epidemics in 1696-1697 killed a quarter to a third of the population of fewer than 450 000. The latest bottleneck, due to consecutive years of crop failure, occurred as late as in 1866-1868 killing 15 % of the population. Official population statistics were founded as early as 1749. Thus, the population was about 480 000 in 1750, 1.6 millions in 1850 and 4 millions in 1950. Only during the most recent 10-12 generations, the number of people has grown rapidly to the present day 5.2 millions. The influence of the bottleneck phenomenon is similar to that of the founder effect. In both cases, genetic drift favours some genes and removes others. (Nevanlinna 1972, Norio et al. 1973, Norio 2003a).

2.5.4.1 The Finnish Disease Heritage

Genetic drift worked in the population sub-isolates modifying the gene pool resulting in enrichment of mainly recessive diseases, denoted as The Finnish Disease Heritage (FDH). This concept was first introduced in 1973 and it refers to monogenic diseases that are more prevalent in the Finnish population than in other populations (Norio et al. 1973). On the other hand, some diseases that are common elsewhere are extremely rare in Finland. Currently 36 rare hereditary diseases belong to the FDH, 32 of which are autosomal recessive, two are autosomal dominant and two are X-linked disorders (Norio 2003c). The gene has been mapped in 33 and characterized in 27 of the disorders belonging to the FDH (Norio 2003c). In the three non-mapped disorders, the number of known families is still small. Diseases of the FDH are typically caused by one major mutation originating from a common ancestor, i.e. a founder mutation, but for some diseases also other minor mutations are known (Norio 2003c). Regional clusters of some of the FDH diseases still exist, reflecting the regional sub-isolates characteristic for a founder population (Peltonen et al. 1999). Founder mutations are characterized by linkage disequilibrium (LD) and significant LD extends over large genomic regions around the disease alleles of the FDH, varying between 2 and 13 cM (Peltonen et al. 1999).

Outside of the FDH, dominant founder mutations in cancer predisposition syndromes, e.g. in hereditary nonpolyposis colorectal cancer, have spread and become highly enriched and specific for certain geographical areas (Nyström-Lahti et al. 1994, Moisio et al. 1996). Similar effect can be seen with *BRCA1* and *BRCA2* mutations, although these mutations are more various (Sarantaus et al. 2000). Thus, low-penetrance susceptibility genes and alleles can be expected to be enriched in a population like the Finns. Genes for several diseases of the FDH have been discovered in analyses exploiting the extent of LD (Järvelä et al. 1991, Hästbacka et al. 1992, Kere et al 1993, Höglund et al. 1995, Varilo et al. 1996, Visapää et al. 1999). The Eastern Finnish population being a regional subpopulation is an especially suitable material for LD analysis (Soininen 1981, Pirinen 1982).

2.6 Methods for mutation detection

However promising a candidate-gene for a hereditary predisposition is it must be shown to be mutated in affected people. Autosomal dominant disease-causing mutations are distinguished from polymorphisms by their functional consequences, their cosegregation with the disease in a family, and their absence in unaffected and unrelated individuals. In some circumstances the identification and interpretation of the mutations may, however, be more difficult. These are unsuspected locus heterogeneity, mutational homogeneity, mutations may not be unambiguously pathogenic or mutations are difficult to find, e.g. *BRCA1* and *BRCA2* genes are large and screening them for mutations is laborious. Mutation testing methods can be divided into two groups: 1) Mutation detection methods for testing samples for known mutations and 2) Mutation screening methods for screening samples for (unknown) mutations in an entire candidate-gene. (Strachan and Read 2003).

2.6.1 Heteroduplex analysis, HA

Heteroduplex analysis is suitable for general mutation screening. It is based on heteroduplex formation when complementary single DNA strands of a wild-type allele

and a mutant allele are allowed to hybridize. Heteroduplexes are formed when PCR products are denatured and then renaturated by cooling down slowly. The detection of heteroduplexes is based on their different mobility on nondenaturing polyacrylamide electrophoresis gels compared to wild-type (or mutant-mutant) homoduplexes. Heteroduplexes have lower electrophoretic mobility and they can be detected as extra slow-moving bands. After electrophoresis the bands are made visible by e.g. ethidium bromide staining. Insertions, deletions and most single base substitutions are detectable in fragments of ≤ 200 bp. HA method does not detect homozygous mutations and it does not reveal the precise position of the mutation. (Keen et al. 1991, White et al. 1992).

2.6.2 Single-strand conformation polymorphism, SSCP/Conformation-sensitive gel electrophoresis, CSGE

SSCP/CSGE can be used to detect known mutations or to screen for unknown mutations. Like HA, SSCP is based on different electrophoretic mobility between mutant and wild-type alleles. In SSCP the gene-specific PCR products are denatured to be single-stranded and run on a nondenaturing polyacrylamide gel. Single-strand molecules adopt conformations that are dependent upon their sequence. In 80-100 % of cases a single-base change is sufficient to cause altered electrophoretic mobility. Radiolabelled primers or silver staining can be used for detecting the bands on gels. Control samples must be run so that differences from the wild-type pattern can be noticed. SSCP is simple and adequately sensitive, but is inefficient for fragments longer than 200 bp, and does not reveal the nature or position of any mutation detected. The precise pattern of bands seen is very dependent on details of the electrophoresis conditions. (Orita et al. 1989, Spinardi et al. 1991, Sheffield et al. 1993).

2.6.3 Protein truncation test, PTT

PTT is a specific test for frameshift, splice-site or nonsense mutations that cause a truncated protein product (Roest et al. 1993, van der Luijt et al. 1994). In certain genes, e.g. *BRCA1* and *BRCA2*, missense mutations are seldom found and are probably usually

nonpathogenic. For such genes the PTT has several advantages. It conveniently ignores the nonpathogenic (nontruncating) silent or missense base substitutions, and it reveals the approximate location of any mutation. Also, in PTT analysis longer fragments can be analysed compared to HA and SSCP.

In the procedure coding sequence without introns (cDNA made by RT-PCR or large exons in genomic DNA) is PCR amplified using a special forward primer that includes (in addition to the gene specific sequence) a T7 promoter sequence and a eukaryotic translation initiator with an ATG start codon, designed so that the sequence amplified reads in-frame from the ATG. The PCR product is put into a coupled *in vitro* transcription-translation system, which uses the T7 promoter to make mRNA and the translation initiator to translate it. The protein product is run out on a SDS-polyacrylamide gel (PAGE). The protein products can be visualised by using autoradiography e.g. incorporating a radioactive labeled (S^{35}) amino acid in the translation reaction. Truncating mutations result in shorter products than the wild-type protein and the size of the fragment reveals the position of the mutation. (Roest et al. 1993, van der Luijt et al. 1994).

2.6.4 Restriction fragment length polymorphism, RFLP

Restriction fragment length polymorphism analysis, RFLP, is based on the variation on the length of DNA fragments cut by different restriction enzymes. A mutation may create a cleavage site for a restriction enzyme which can be used in detecting the mutation. By this technique mutated alleles may be differentiated from wild-type alleles by analysis of patterns derived from cleavage of their DNA. If two alleles differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. (Strachan and Read 2003).

2.6.5 Sequencing

Sequencing can be used to further characterize and verify a mutation found in analyses done by other mutation screening methods. Sequencing can also be used to screen for

mutations but it can be more laborious than usual mutation screening methods if the mutations searched for are not known in advance. The two best-known DNA-sequencing techniques are the enzymatic (dideoxy-mediated chain-termination) method of Sanger et al. (1977) and the chemical degradation method of Maxam and Gilbert (1977). Of these two Sanger's method is more widely used.

3. AIMS OF THE STUDY

The general aim of this study was to investigate the genetic background of familial breast/ovarian cancer and sporadic breast cancer in Eastern Finnish population. Since the population in this area has been relatively stable and genetically isolated, founder effect in the hereditary forms of breast/ovarian cancer could be expected. The frequency and type of germ-line mutations in known high-risk breast/ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, were evaluated in Eastern Finnish breast/ovarian cancer families. In addition, the autosomes were screened to identify new chromosomal regions and genes on them as genetic risk factors for sporadic breast cancer in the Eastern Finnish population. Due to the character of a founder population, linkage disequilibrium could be utilized.

The specific aims of the study were:

I To screen 36 breast/ovarian cancer families for *BRCA1* and *BRCA2* germ-line mutations for evaluation of the contribution of these genes in Eastern Finland.

II To analyse the novel *BRCA2* exon 11 4088insA mutation in an Eastern Finnish family with multiple breast/ovarian cancers and favourable clinical course.

III To study the effect of *BRCA1* 4216-2nt A→G splice acceptor site mutation on *BRCA1* protein product.

IV To find new low-penetrance genetic risk factors for Eastern Finnish sporadic breast cancer by using autosome-wide microsatellite scan for linkage disequilibrium-based association in a case-control material.

V To identify the putative breast cancer risk-associated gene on 22q12-q13 region by single nucleotide polymorphism (SNP) association analysis.

4. MATERIAL AND METHODS

4.1 Study subjects

4.1.1 Breast and ovarian cancer families (I-III)

The pedigrees in the study are from the Kuopio University Hospital district and of Eastern Finnish origin. Of the below described 516 new breast cancer cases diagnosed between April 1990 and December 1995, 102 patients were selected on the basis of some positive family history of cancer, i.e. breast or ovarian cancer in one first degree relative and in one other relative, according to the interview data collected by the Kuopio Breast Cancer Project (Männistö et al. 1996). The criteria were not strict at this point. The 102 selected patients were invited for an additional interview, gynaecologic ultrasound examination and were offered preliminary genetic counseling. They were also asked to fill in a questionnaire to further determine their family history of cancer. A filled questionnaire was returned by 85/102 patients and 82/102 patients participated in the ultrasound examination and agreed to the interview. On the basis of the data arising from the pedigrees, 15/82 families were selected for the germ-line *BRCA1* and *BRCA2* gene mutation screening study. The selection criteria were a minimum of three first-degree relatives with breast or ovarian cancer. However, 7/15 families with less than three first-degree relatives with breast or ovarian cancer were included in the study, due to history of cancer at early age, bilateral breast cancer or several cases of breast/ovarian cancer in the second or third-degree relatives.

The questionnaire was also sent to ovarian cancer patients treated in Kuopio University Hospital between January 1993 and October 1997. Out of over 90 Eastern Finnish pedigrees, 14 were selected to be included in the study following discussions with the index patients. Criteria for the ovarian cancer families were at least two cases of ovarian cancer in the family. Seven other families fulfilling the criteria were also included in this study. Six of these seven families were identified through genetic counseling at the local Cancer Society policlinic, and one was identified by the clinical oncologist of the research group in routine clinical consultations. Thus, altogether 36 breast and ovarian cancer families were included in the germ-line mutation screening

study (Tables 17 and 18). In addition to breast and ovarian cancers, other cancers were also reported among first degree relatives in 2/3 of the families.

Table 17. Characteristics of the studied breast/ovarian cancer families.

	No. of cancer in family		
Family	**Breast**	**Ovarian**	**Other cancers in family**
BR10	7		gastric, colon x3, lung x2
BR14/16	6		gastric, unknown cancer
BR17	6 (1 bilat.)		skin melanoma
BR13	5		unknown cancer, lung
PS20	3 (1 bilat.)		Wilms tumour, brain, colon
BR02	3		
BJ01	3 (1 bilat.)		leukaemia, unknown cancer x3
BR12	4 (1 bilat.)		lung
BR08	3 (1 bilat.)		lung
BR05	3		
PS04	2		skin melanoma, liver, thyroid, oesophageal, lymph node, endometrial
PS19	2 (1 bilat.)		endometrial x2, unknown cancer x2
BR01	2		endometrial
BR04	2		melanoma
BR11	2		lung, colon
BR15	2		unknown cancer x2
OV03	5	2	brain
BR09	4	3 (1 bilat.)	rectal, prostate
PS02	4	1	gastric x2
OV30	2	3	pancreas, gastric, lip
OV01	2	2	
OV85	3	1	lymphoma, leukaemia, colon
BR06	4	1	chloledocal, gastric, lung, kidney
BR18	2	2	prostate
BR03	2	1	prostate, bladder, oesophageal or laryngeal, liver, unknown cancer
BR07	1	1	gastric x2, endometrial x3
OV28	2	1	lung, colon
OV73	2	1	melanoma, gastric x2, cervix, thyroid, oesophageal
OV37	1	1	colon, lung
OV84	1	1	
OV20		3	unknown cancer x3
OV22		3	lip, lung x3, gastric, lymphoma, laryngeal
OV36		2	pancreas, lung x3, endometrial, lip, cervix, gastric, colon
OV38		2	endometrial x2, bone, prostate, neck, leukaemia, lymphoma
OV77		1	neuroblastoma
OV33		1	bladder, gastric

EDTA blood samples were collected from the breast and ovarian cancer patients participating in the study and from their relatives who were willing to participate and

who gave their consent. Genomic DNA was extracted from peripheral blood lymphocytes as described below.

4.1.2 Population-based breast cancer case-control material (IV-V)

The population-based sample material is carefully selected from the province of Northern Savo in the late-settlement area of Eastern Finland. This study is an extension of the Kuopio Breast Cancer Project, through which the cases for this study were ascertained (Männistö 1999). The material was collected from 1 919 women who came to Kuopio University Hospital (hospital district of 750 000 people) between April 1990 and December 1995 with any suspected breast disease for breast examination. Of these women 516 were diagnosed and histologically confirmed to have breast cancer. These women with breast cancer were asked to participate in the study and only twelve refused to participate. Thus, the participation/cooperation rate was 98 % (504/516). The recruitment protocol missed 88 women diagnosed with breast cancer in Kuopio University Hospital catchment area during the collection period, 51 of which were private patients, 11 were missed during the nurses' one-month strike in 1995 and 26 were treated elsewhere. Therefore the total contact rate was 85 % (516/604) and overall response rate 83 % (504/604). Thus, the sample is a representative collection of unselected typical breast cancer cases from Kuopio area. A proper DNA sample was available from 497 breast cancer cases for the association study.

In addition to breast cancer cases, the Kuopio Breast Cancer Project material includes randomly selected and individually matched age (within ± 5 years) and area-of-residence (urban/rural) controls from the National Population Register for each case of breast cancer (Männistö 1999). Seventy-two percent (359/497) of the cases and 73 % (334/458) of the population controls included in study V were born in the Province of Northern Savo. (Table 18). Each participant gave informed written consent. EDTA blood samples were collected from the breast cancer cases and controls participating in the study. Genomic DNA was extracted from peripheral blood lymphocytes as described below.

4.1.2.1 Cases and controls in the autosome-wide scan for LD (IV)

For the autosome-wide scan for linkage disequilibrium with breast cancer 49 cases and 50 controls selected from the above described population-based material were used. Forty-nine breast cancer cases who did not have a strong family history of cancer according to the initial interview and who were born around Kuopio in four rural communities whereto moving has been minimal were selected for the study IV (Table 18). The included 50 controls are matched for age and long-term area-of-residence with the cases. Age of onset of breast cancer ranged between 30 and 82 years, median age of onset being 54.0 years. Seventy-three percent of the cases had breast cancer at or after the age of 50 years. The median age of the controls was 54.5 years (range 30-76 years).

4.1.2.2 Cases and controls in refining of the breast cancer-associated region on 22q12-q13 (V)

For the further study of chromosome 22q12-q13 breast cancer association we had DNA available from 497 breast cancer cases and 458 age and long-term area-of-residence matched controls. The selected cases represent 96 % of the total of 516 breast cancers diagnosed in Kuopio University Hospital between 04/90 and 12/95.

Table 18. Summary of study subjects used in each study.

	Study I	**Study II**	**Study III**	**Study IV**	**Study V**
Study design	Familial	Familial	Familial	Case-control	Case-control
Cancer	Breast/ovarian	Breast/ovarian	Breast	Breast	Breast
No. of studied individuals	89, in 36 families	11, in 1/36 family	20, in 2/36 families	49 + 50	497 + 458 (subsample 280 + 257)

4.2 Extraction of DNA from blood lymphocytes (I-V)

EDTA blood samples were collected from the familial breast and ovarian cancer patients participating in the study and from their relatives who were willing to participate and who gave their consent (I-III), and from the sporadic, population-based breast cancer patients and controls participating in the study (IV, V). Genomic DNA

was extracted from peripheral blood lymphocytes using standard chloroform-phenol extraction method (Vandenplas et al. 1984).

4.3 *BRCA* gene mutation detection (I-III)

In study I the DNA samples were screened for germ-line mutations in the coding region and exon-intron boundaries of the *BRCA1* and *BRCA2* genes by heteroduplex analysis (Ganguly et al. 1993), protein truncation test (PTT) (Roest et al. 1993) and direct sequencing. Variants found in PTT and HA were confirmed by sequencing. In study II the *BRCA2* 4088insA mutation was detected in PTT analysis and confirmed by sequencing. In study III the *BRCA1* 4216-2nt A→G mutation was confirmed, after sequencing, with restriction fragment length polymorphism analysis (RFLP) using the restriction enzyme MseI (New England BioLabs, Beverly, MA, USA). Additionally, in study III the effect of the mutation on the intron splice-site was confirmed by cDNA analysis.

4.3.1 PCR amplification and protein truncation test (PTT) of *BRCA1* and *BRCA2* exon 11 (I, II)

The exon 11 of *BRCA1* and *BRCA2* gene were screened for germ-line mutations by protein truncation test (PTT) (Roest et al. 1993, Hogervorst et al. 1995). First, exon 11 of both genes was PCR-amplified from genomic DNA in three overlapping segments with a T7 promoter sequence attached to the 5' end of the forward primers (Plummer et al. 1995, Johannsson et al. 1996, Tavtigian et al. 1996) (Table 19, Figure 8). Annealing temperature for each primer was 56-58°C and Taq DNA polymerase (Promega, Madison, WI, USA) was used. The PCR products were used as a template for in vitro transcription translation reaction. For PTT the TNT T7 Coupled Reticulocyte Lysate System (Promega) was used according to manufacturer's instructions. The protein products were detected by autoradiography. First they were separated on a 12 % SDS-PAGE minigel (BioRad, Hercules, CA, USA), then gels were fixed with 1 M sodium acetate and dried. X-ray films were exposed in -70°C for 1-5 days and developed.

Table 19. Sequences of the primers used in PTT analysis of *BRCA*1 and *BRCA2* gene exon 11.

Primer name	Sequence 5' → 3'	Location (nt) in cDNA
BR1-AF	T7-CTGCTTGTGAATTTTCTG	790 - 807
BR1-AR	CATGAGATCTTTGGGGTC	2355 - 2372
BR1-BF	T7-AGCAGAATGGTCAAGTG	1738 - 1754
BR1-BR	GTCCAGTTTCGTTGCCTC	2995 - 3012
BR1-CF	T7-TCAAGGTTTCAAAGCGCCAG	2701 - 2720
BR1-CR	GACGTCCTAGCTGTGTGAAG	4215+185nt in intron 11
BR2-AF	T7-GTTTATTGCATTCTTCTGTG	2138 - 2157
BR2-AR	TGACTTCCTGATTCTTCTAA	3580 - 3599
BR2-BF	T7-GTGTAAAGCAGCATATAAAAAT	3101 - 3122
BR2-BR	TTCGGAGAGATGATTTTTGTC	5410 - 5430
BR2-CF	T7-GCCTTAGCTTTTTACACAA	5230 - 5248
BR2-CR	CCCCCAAACTGACTACACAA	7069+135nt in intron 11

T7 promoter sequence: 5'-GGATCCTAATACGACTCACTATAGGAACAGACCACCATGG-3'

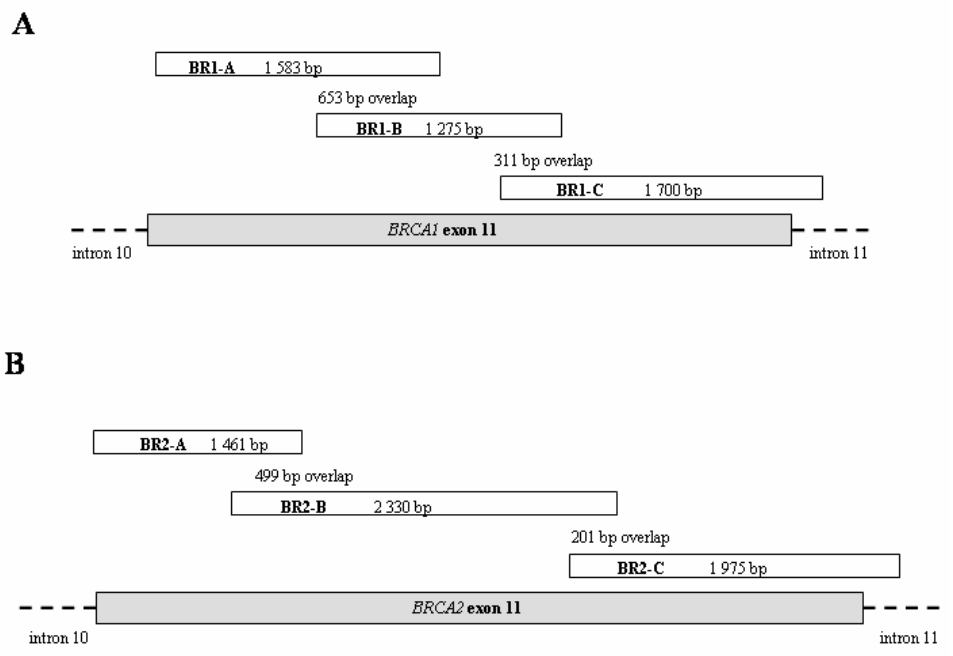


Figure 8. Exon 11 of *BRCA1* and *BRCA2* genes were analysed in three overlapping fragments using PTT. Size of each fragment (BR1-A - BR2-C) is shown in base pairs (bp). A) Schematic presentation of *BRCA1* exon 11. B) Schematic presentation of *BRCA2* exon 11.

4.3.2 Heteroduplex analysis of *BRCA1* and *BRCA2* genes (I, III)

In study I and III exons 2, 3, 5-10 and 12-24 of the *BRCA1* gene were amplified from genomic DNA using the primers described by Friedman et al. (1994), Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) and annealing temperatures 56-63°C (Table 20). In study I exons 2 and 12 of the *BRCA2* gene were amplified using the primers described by Couch et al. (1996) and exons 3-9 and 13-27B using primers described by Friedman et al. (1997) (Table 21). Annealing temperatures were 47-59°C and Dynazyme DNA polymerase was used. An aliquot of 3-8 µl of the PCR product was used for the DNA pairing reaction of the heteroduplex (HA) analysis. The sample was first denatured in 98°C for 5 minutes and then it was renaturated by cooling the heatblock slowly to 37°C. The hetero- and homoduplexes are formed during the slow renaturation. After cooling, 6 µl of loading buffer (30 % glycerol, 0.25 % bromophenol blue, 0.25 % xylencyanol) was added into sample tube and then briefly centrifuged. The samples were analyzed on 10 % CSGE gel. Electrophoresis was carried out with 150V voltage for 12-15 hours depending on the size of the exon analysed. After electrophoresis the gels were either silver stained and dried or ethidiumbromide (EtBr) stained and photographed.

4.3.3 Sequencing (I-III, V)

In study I the exon 10 of the *BRCA2* gene was screened for mutations by direct sequencing. The exon 10 was PCR amplified in two overlapping fragments using Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) according to manufacturer's instructions. The forward and reverse primers were 5'- TGTTTCT ATGAGAAAGGTTGTGAGA-3' and 5'- TTGCCTGCTTTACTGCAAGA-3' for the first fragment, and 5'- TAAATGGAGCCCAGATGGAG-3' and 5'- AAAACACA GAAGGAATCGTCATC-3' for the second fragment. A 1.0-1.5-µl aliquot of the PCR product was included in the sequencing reactions using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to manufaturer's instructions. Sequences were run on an ABIPRISM 3100 Genetic Analyzer (Applied Biosystems)

Table 20. Primers used in the heteroduplex analysis (HA) of *BRCA1* gene.

Primer pair	Exon size (bp)	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'	Fragment size(bp)
Exon 2	100	GAAGTTGTCATTTTATAAACCTTT	TGTCTTTTCTTCCCTAGTATGT	254
Exon 3	54	TCCTGACACAGCAGACATTTA	TTGGATTTTCGTTCTCACTTA	349
Exon 5	78	CTCTTAAGGGCAGTTGTGAG	TTCCTACTGTGGTTGCTTCC	238
Exon 6	89	CTTATTTTAGTGTCCTTAAAAGG	TTTCATGGACAGCACTTGAGTG	166
Exon 7	140	CACAACAAAGAGCATACATAGGG	TCGGGTTCACTCTGTAGAAG	228
Exon 8	105	TGTTAGCTGACTGATGATGGT	ATCCAGCAATTATTATTAAATAC	267
Exon 9	47	CCACAGTAGATGCTCAGTAAATA	TAGGAAAATACCAGTTTCATAGA	211
Exon 10	76	TGGTCAGCTTTCTGTAATCG	GTATCTACCCACTCTCTTCTTCAG	242
Exon 12	87	GTCCTGCCAATGAGAAGAAA	TGTCAGCAAACCTAAGAATGT	265
Exon 13	174	AATGGAAAGCTTCTCAAAGTA	ATGTTGGAGCTAGGTCCTTAC	320
Exon 14	127	CTAACCTGAATTTACACTTACA	GTGTATAAATGCCTGTATGCA	312
Exon 15	191	TGGCTGCCCAGGAAGTATG	AACCAGAATATCTTTATGTAGGA	338
Exon 16	311	AATTCTTAACAGAGACCAGAAC	AATTCTTAACAGAGACCAGAAC	450
Exon 17	88	GTGTAGAACGTGCAGGATTG	TCGCCTCATGTGGTTTTA	264
Exon 18	80	GGCTCTTTAGCTTCTTAGGAC	GAGACCATTTTCCCAGCATC	351
Exon 19	37	CTGTCATTCTTCCTGTGCTC	CATTGTTAAGGAAAGTGGTGC	251
Exon 20	86	ATATGACGTGTCTGCTCCAC	GGGAATCCAAATTACACAGC	401
Exon 21	55	AAGCTCTTCCTTTTTGAAAGTC	GATGAGAAATAGAATAGCCTCT	298
Exon 22	75	TCCCATTGAGAGGTCTTGCT	GAGAAGACTTCTGAGGCTAC	297
Exon 23	60	CAGAGCAAGACCCTGTCTC	ACTGTGCTACTCAAGCACCA	255
Exon 24	125	ATGAATTGACACTAATCTCTGC	GTAGCCAGGACAGTAGAAGGA	280

Table 21. Primers used in the heteroduplex analysis (HA) of *BRCA2* gene.

Primer pair	Exon size (bp)	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'	Fragment size (bp)
Exon 2	106	TTCCAGGAGATGGGACTGAATTAG	TGGGTTTTTAGCAAGCATTTTTTAG	302
Exon 3	249	GATCTTTAACTGTTCTGGGTCACA	CCCAGCATGACACAATTAATGA	423
Exon 4	109	AGAATGCAAATTTATAATCCAGAGTA	AATCAGATTCATCTTTATAGAACAAA	249
Exon 5&6	51&41	TGTGTTGGCATTTTAAACATCA	CAGGGCAAAGGTATAACGCT	453
Exon 7	115	CCTTAATGATCAGGGCATTTC	CAACCTCATCTGCTCTTTCTTG	214
Exon 8	50	GCCATATCTTACCACCTTGTGA	AGGTTTAGAGACTTTCTCAAAGGC	406
Exon 9	112	CTAGTGATTTTAAACTATAATTTTTG	GTTCAACTAAACAGAGGACT	164
Exon 12	99	AATTGACATTGAAGACTGACTTTACTC	AGCACTTTGGAGAGGCAGG	374
Exon 13	73	GCATCCGTTACATTCACTGAAA	ACGGGAAGTGTTAACTTCTTAACG	310
Exon 14A	428	ACCATGTAGCAAATGAGGGTCT	GCTTTTGTCTGTTTTCCTCCAA	391
Exon 14B	428	CACAGAGTTGAACAGTGTGTTAGG	GGGCTTTAAAATTACCACCACC	297
Exon 15	182	GGCCAGGGGTTGTGCTTTTT	AGGATACTAGTTAATGAAATA	314
Exon 16	188	TTTGGTAAATTCAGTTTTGGTTTG	AGCCAACTTTTTAGTTCGAGAG	395
Exon 17	171	CAGAGAATAGTTGTAGTTGTTGAA	AGAAACCTTAACCCATACTGC	306
Exon 18A	355	GATCCACTATTTGGGGATTGC	GATCTAACTGGGCCTTAACAGC	457
Exon 18B	355	GCAGATACCCAAAAAGTGGC	TCTGGACCTCCCAAAAACTG	384
Exon 19	156	AAGTGAATATTTTTAAGGCAGTT	TATATGGTAAGTTTCAAGAAT	296
Exon 20	145	CACTGTGCCTGGCCTGATAC	ATGTTAAATTCAAAGTCTCTA	296
Exon 21	122	GGGTGTTTTATGCTTGGTTCT	CATTTCAACATATTCCTTCCTG	304
Exon 22	199	AACCACACCCTTAAGATGAGC	GGGCATTAGTAGTGGATTTTGC	455
Exon 23	164	ACTTCTTCCATTGCATCTTTCTCA	AAAACAAAACAAAAATTCAACATA	290
Exon 24	139	GCAGCGACAAAAAAAACTCA	ATTTGCCAACTGGTAGCTCC	365
Exon 25	305	GCTTTCGCCAAATTCAGCTA	TACCAAAATGTGTGGTGATGC	426
Exon 26	147	GTCCCAAACTTTTCATTTCTGC	GGAGCCACATAACAACCACA	379
Exon 27A	609	CTGTGTGTAATATTTGCGTGCT	GCAAGTTCTTCGTCAGCTATTG	495
Exon 27B	609	GAATTCTCCTCAGATGACTCCA	TCTTTGCTCATTGTGCAACA	417

and analysed using Sequencing Analysis and Sequence Scanner v1.0 softwares (Applied Biosystems).

When aberrant mobility on heteroduplex gels or PTT protein product differing from the wild-type protein was detected in studies I-III, the variants were reamplified from genomic DNA and sequenced and analysed using dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and AbiPrism 310 Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions. The primers used for HA in study I and III were used also for sequencing the fragments. For sequencing the variant in PTT in study I and II new primers were designed (BR2-05F 5'- ATGAAGTGGGGTTTAGGGGC-3' and BR2-05R 5'-ATTTTGCTCCGTTTTAGT AG-3'). In study III primers SPL1F 5'-ACCGTTGCTACCGAGTGTCT-3' and SPL1R 5'- AGTTCAGCCATTTCCTGCTG-3' and the above technique and equipment were used for sequencing of the purified cDNA.

In study V the variants from DHPLC analysis were re-amplified and sequenced using the primers used in DHPLC analysis and AbiPrism Big Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq DNA Polymerase (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Samples were analysed on AbiPrism 377 DNA sequencer and Sequencing Analysis 3.3 software (Applied Biosystems).

4.3.4 RFLP analysis, extraction of RNA, and cDNA analysis of the *BRCA1* 4216-2nt A to G mutation (III)

In addition to HA and sequencing, the 4216-2nt A→G splice-site mutation was confirmed by restriction fragment length polymorphism (RFLP) analysis from genomic DNA. A 265-bp-fragment was PCR amplified using the primers (12F and 12R) used in HA and the fragment was digested using 5 units of the restriction enzyme MseI (New England BioLabs, Beverly, MA, USA) in conditions recommended by the manufacturer. The recognition sequence for MseI is T/TAA. Two cleaving sites for MseI locate in the wild-type allele, but only one in the 4216-2nt A→G mutated allele. Thus, digested fragments are 82, 83 and 100 bp in non-carriers and 100 and 165 bp in

4216-2nt A→G mutation carriers. After digestion the fragments were separated on a 2.5 % LE agarose gel (FMC Bio Products), visualised by EtBr staining and photographed.

For study III also a fresh EDTA blood sample was drawn from *BRCA1* exon 12 4216-2nt A→G splice-site mutation carrier in one of the families. Total RNA was extracted from the blood lymphocytes using a Trizol LS Reagent (GibcoBRL Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was performed from approximately 0.5 μg of total RNA using Titan™ One Tube RT-PCR System (Boehringer Mannheim, Mannheim, Germany) and primers SPL1F and SPL1R designed with the Primer3 program available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. SPL1F is located in *BRCA1* exon 11 at the nucleotides from 3855 to 3874 and SPL1R in exon 13 at the nucleotides from 4341 to 4360. The size of the normal cDNA fragment amplified with SPL1F and SPL1R primers is 506 bp and mutated fragment 507 bp. (Figure 9). The RT-PCR conditions were 50° for 30 min, 94° for 2 min, 46 cycles of 94° for 30 sec, 55° for 30 sec and 68° for 2 min and the final step 68° for 5 min. After amplification the size of the cDNA product was checked on a 2 % LE agarose (FMC Bio Products, Rockland, ME, USA) gel and purified with QIAquick PCR Purification kit (QIAGEN, Hilden, Germany). The purified cDNA product was used for sequencing as described above in 4.3.3.

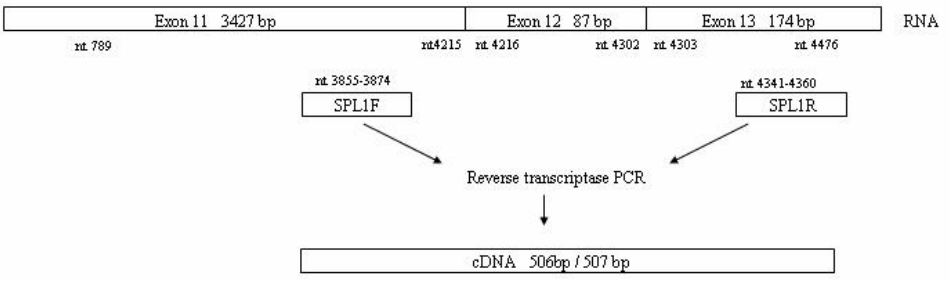


Figure 9. Schematic presentation of reverse transcriptase (RT) PCR amplification for *BRCA1* 4216-2nt A→G splice-site mutation analysis. Length of *BRCA1* exons 11-13 and location of primers is shown in nucleotides (nt). The size of amplified cDNA fragment is 506 bp and in mutation carriers 507 bp.

4.3.5 Analysis of mutation-associated haplotypes (I-III)

Microsatellite markers *D13S260*, *D13S1701* and *D13S267* were genotyped for the identification of the *BRCA2* gene mutation-associated haplotypes (Table 22). The microsatellites were PCR amplified with fluorescent-labelled primers using standard procedures (Sheffield et al. 1995) and analysed using an automated ABIPrism 310 Genetic Analyzer (Applied Biosystems), GeneScan 2.1 software and internal standard (see below at 4.4). Haplotypes were constructed manually utilizing the pedigree data. The location and distance between markers (kb and cM) was obtained from the human Genome Browser Gateway at the University of California at Santa Cruz (UCSC) (http://genome.ucsc.edu).

Table 22. Microsatellites used for *BRCA2* mutation-associated haplotypes.

Marker	Location on Chr.13	Distance in kb	Genetic location (cM)
cen	0		
D13S260	31334758 - 31335105		23.65
BRCA2 (gene)	31787617 - 31871805	452.512	
D13S1701	32042530 - 32042819	170.725	
D13S267	33162119 - 33162387	1119.300	26.87
tel	114142980		

4.4 Genotyping of microsatellites in the autosome-wide scan for LD (IV)

In study IV, 366 microsatellite markers from the 6th version of the Weber lab marker set (http://research.marshfieldclinic.org/genetics/GeneticResearch/sets/scrset6.txt) were genotyped in the first-stage autosome-wide scan for LD with breast cancer. The distance between markers span approximately 10 cM (range 1-20 cM) in the genome and the average heterozygosity of the markers was 0.76. The microsatellite repeats were PCR amplified from genomic DNA in single or multiplex reactions with fluorescent-labelled primers using standard procedures (Sheffield et al. 1995). PCR products from several microsatellites were pooled individually for each case and control and an internal GeneScan-500 [TAMRA] size standard (Applied Biosystems) was added into each sample. The size of the microsatellites was determined with an automated AbiPrism 310 Genetic Analyser (Applied Biosystems) and analysed using the GeneScan 2.1 software (Applied Biosystems) according to the size standard.

The number of samples was designed so that it was possible to run all controls, 45 of the cases and a blank control in one run on 96-well plate using AbiPrism 310. This was done to minimize possible variations in the sample running conditions (e.g. by using the same capillary for all samples). The remaining four of the cases were run first on another run to re-check that the reaction conditions for the marker are fine.

When linkage disequilibrium with breast cancer was observed, additional markers close to those with significant (or near significant) *P*-values were selected from the Genome Database (http://www.gdb.org/) for the second-stage scan (Table 23). Altogether 69 additional markers were genotyped. Intermarker distances and order were obtained from the databases at the Sanger Institute (http://www.sanger.ac.uk), the Human Genome Browser Gateway at the UCSC (http://genome.ucsc.edu), Cedar Genetics at the University of Southampton (http://cedar.genetics.soton.ac.uk) and Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics). In the second-stage scan an AbiPrism 377 DNA Sequencer (Applied Biosystems) and GeneScan 2.1 (Applied Biosystems) software were used for size determination.

Table 23. Additional markers used in the second-stage of the genome-wide scan.

Chromosome 1	**Chromosome 7**	**Chromosome 13**	**Chromosome 20**
D1S2800	*D7S519*	*D13S280*	*D20S445=D20S79*
Chromosome 2	*D7S2422*	*D13S1265*	*D20S887*
D2S363	*D7S2423*	**Chromosome 14**	*D20S196*
D2S1277	**Chromosome 8**	*D14S1016*	**Chromosome 21**
D2S2289	*D8S1008*	*D14S1054*	*D21S1255*
D2S2358	*D8S1694*	**Chromosome 16**	**Chromosome 22**
D2S2248	**Chromosome 9**	*D16S3091*	*D22S277*
D2S163	*D9S157*	*D16S488*	*D22S1142*
D2S2202	*D9S1684*	**Chromosome 17**	*D22S924*
D2S2253	**Chromosome 10**	*D17S1810*	*D22S283*
Chromosome 3	*D10S580*	*D17S1289*	*D22S426*
D3S3630	*D10S607*	*D17S799*	*D22S1177*
D3S1229=D3S1246	*D10S201*	*D17S1785*	*IL2RB*
D3S3725	*D10S523*	*D17S1820*	*D22S1156*
D3S3520	**Chromosome 11**	*D17S941*	*D22S272*
Chromosome 4	*D11S1302*	*D17S1302*	*D22S284*
D4S2975	*D11S1893*	**Chromosome 19**	*D22S279*
D4S1615	*D11S1347*	*D19S198*	*D22S270*
D4S1575	*D11S897*	*D19S420*	
Chromosome 6	**Chromosome 12**	*D19S559*	
D6S1660	*D12S1659*	*D19S219*	
D6S980		*D19S545*	
D6S1599			

4.5 Validating and selecting of SNPs for the refining of the breast cancer-associated region on 22q12-q13 (V)

SNPs in genes in the 550-kb region between microsatellites *D22S426* and *D22S445* on chromosome 22q12-q13 were searched from databases (http://www.sanger.ac.uk, http://www.ncbi.nlm.nih.gov/SNP and http://snp.cshl.org/db/snp) and assays were designed for confirming the frequencies of 26 suggested SNPs and a small deletion on denaturing high-pressure liquid chromatography (DHPLC). Also the coding, 5'UTR and 3'UTR sequences of one of the genes in the associated region (*RABL4*) were screened for sequence variations using DHPLC. (Table 24). For these validations genomic DNA from 48 anonymous British samples and 48 of the 49 Eastern Finnish breast cancer cases used in the genome-wide LD scan were used. PCR amplifications for DHPLC analysis were carried out using: 1x GeneAmp PCR Gold buffer (Applied Biosystems), 0.16mM dNTP, 1.5-1.6 mM MgCl, 0.3-0.4 mM primers, 1.25-2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 20 ng genomic DNA in a 60-µl reaction and standard protocols. After amplification the samples were denatured and slowly renatured using 3 min denaturation at 95°C followed by cooling down to 25°C one degree per min. PCR amplified samples were analysed on WAVE DHPLC equipment (Transgenomics, Crewe, UK) according to manufacturer's instructions and using specific conditions for each fragment. The variants from DHPLC analysis were re-amplified and sequenced as described above in section 4.3.3.

Ten of the validated SNPs were selected for genotyping in 497 sporadic breast cancer cases and 458 matched controls (material described in 4.1.2.2). The SNP selection was based on the allele frequency (rare allele frequency > 0.1), location and distance between the SNPs (region covered as evenly as possible) and the suitability of the SNP to be detected by the 5' nuclease (Taqman®) assays. Also coding SNPs were preferred over intronic SNPs. The selected SNPs cover a 541-kb region on chromosome 22q12-q13 and adjacent SNPs are 0.9-132 kb apart. The selected SNPs are shown in Table 24 and Figure 10.

Artificial templates were prepared for both alleles in each assay for using as controls for the homozygous genotypes in the analysis. Long oligonucleotides were designed for using as primers in the artificial template synthesis (Table 25). For each SNP three

Table 24. Primers used in SNP validation on DHPLC. SNPs selected for genotyping are indicated with an asterisk (*).

GENE	SNP ID	Variation location EXON/SNP	Location of exon or SNP in NT_011520.11	MAF	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'	Fragment size (bp)
CACNG2	***rs738977**	intron 2 T/C	16399443	0.19-0.39	GCCAGTGGAACCCCTTTATT	CCTTCCAGCTTGCTACCACT	255
RABL4		ex7 + 3'UTR	16544771-16544969‡	NVF	GGCCCTCTGACCCTTCTAAA	AGGCCAAGAGTGGAAGGAG	282
RABL4	***rs3827351**	intron 7 C/A	16547755	0.20-0.43	TCCCTTCCTCCATTCATTTG	TGCTTCTGTGAGCCTCAACT	263
RABL4		ex6	16549464-16549573	NVF	GCTGTCCTTCCAGAGTGTGA	CGACGAGGCAAGTGGAAATA	193
RABL4		ex5	16550479-16550596	NVF	ACTCATCCTGTCCCTCTTGC	TGTGCTCTTGAACCAAAGGA	203
RABL4		ex4	16552698-16552757	NVF	TCACCCCTAAGACAAGTCCAG	AGGAGGTGGTGACAGTCAGG	202
RABL4		ex3	16553866-16553925	NVF	TCCAGAACAAACCCTTCCAC	CCTGGGTAAAGGCATCACTC	203
RABL4		ex2	16554343-16554419	NVF	GCGGGAGATTCTCAAGTCAG	ATGGTGAACCGAGCACTTCT	200
RABL4		ex1	16562234-16562267	NVF	GCTCTTGTCCTCTGGGTACG	TAACTCGCCTTTGGGAGAGA	200
RABL4		5'UTR F&R	16562268-16562636‡	NVF	GGCTATGGTTGCTTGGAGAG	GGCTGATCTCAAGGGTCAGT	292
RABL4		5'UTR F&R2	16562268-16562636‡	NVF	GGCTATGGTTGCTTGGAGAG	ACAAGAGCGGCTGCTAGAGA	371
RABL4		5'UTR F&R3	16562268-16562636‡	NVF	GGCTATGGTTGCTTGGAGAG	TTCAAGCGCAAGTTTTCAGA	464
PVALB	***rs3484**	3'UTR G/A	16587386	0.49	GCTTCTCCAGCATTTTCCAG	CGCCTCTGACTCACTTTCCT	234
PVALB	rs739031	intron 5 G/T	16598664	0.048	TGGCAGGAAACAAACAAACA	AACACGTCACCTCTGCACAC	224
intergenic	rs713953	Z82185 24057 C/T	16623838	0.07-0.156	AATTCTGCTCAGCCTTCCTG	GCTTTCCAAAGAGAAAAGAGCA	218
NCF4	***rs760517**	intron 2 C/T	16649501	0.32-0.48	CCAACAGACATGTTTTGAGCA	CGGAAGTCCCCTGGAATATC	231
NCF4	***rs738148**	intron 8 A/G	16661017	0.31-0.44	TGAAGGAGGTTCTGGGACAG	CGGAAAACAGGAAGGCAATA	260
intergenic	rs735563	AL08637 52259 A/G	16687446	0.22	GTGTCCTCGTTCAGATGCAA	CCCTCTTGAGTCAGGTCAGG	240
CSF2RB	rs16845	exon 7 Glu249Gln	16716958	0.04	GTGGGCACCCACTGAGAG	GAGGAAGCTTGGAGAGAGCA	266
CSF2RB	***rs7285064**	exon 10 C/T Ser426	16720514	0.15-0.375	AGACCGAGACCCTCCAGAAC	GGGATTTTCTGGCAGTGAGA	226
CSF2RB	rs1801122	exon 14 Pro603Thr	16724172	0.02	CAGCTGCCTCAGATCTACCC	CCAGAGGGACCAGTTGCAC	261
CSF2RB	rs1801115 rs131840 rs1801114	exon 14 Gly647Val exon 14 G/A Pro648 exon 14 Val652Met	16724305 16724309 16274319	NA	AGGGTGGGAGCCAGAAGT	ATAGCCACAGGGCTGTCCTT	244
CSF2RB	rs1801116	exon 14 G/A Pro800	16724765	NA	TTTACTCCGGAGCGAGAGAG	TGTGGAGCTCCCTCCAAT	217
TST	not polym.	exon 4 T/C Asp268	16797673	0	GCCTTGGCTGCCTACCTC	CAATTCTAAAAACATGTCATCTCCT	229
TST	rs4764	exon 4 C/T His256	16797709	0.043	CCCCTAAGCTGTGTCCTCTG	GCTTGCCGCAGAGGTAGG	234
TST	rs1803405	exon 4 Glu227Gly	16797797	0	CCCTAAGCTGTGTCCTCTGC	GACCAGGAGCCATCGTACAC	264
TST	rs16363	intron 3 tgttt ins/del	16800400	0.41-0.50	AGGCCCGCTCTACAGAAACT	ACGAGGAACAATGGATCAGC	229
intergenic	***rs25095**	Z73420 37027 G/A	16824543	0.28-0.48	CAGGAGCCAAGGGAGGAG	AAATCAGAGCCCAGTTCCTG	219
KCTD17	rs710184	intron 4 A/G	16843774	0.19	TGTCTCCTTCACCCTTGAGC	ACCACCCCCGCAACATAC	221
TMPRSS6	rs736852	intron 7 T/C	16876720	0.31-0.47	CCTTCTCAGTGCCCAGGTAA	GCCCTGTGTGTACTTTTCTCAA	203
TMPRSS6	***rs733655**	intron 3 T/C	16885566	0.20-0.475	CATCCTTCCACTCGTGAGGT	TGAGTGTCCTCGTCACATCC	232
intergenic	rs228937	AL022314 77611 G/T	16911486	0.180.43	CTTCCCGCTCTGTACCTGAC	CCTTATACCGGGTGCAGATG	231
IL2RB	rs228940	3'UTR C/T	16913835	NA	TTCCCTGACTCTTCAAGTGC	GAAGCCACTGTGGGAAGATG	265
IL2RB	***rs228941**	3'UTR G/C	16914236	0.29	CCCTGCAGCTATTCACCAAT	ACGACACCGGAGGTACAAGT	238
IL2RB	***rs228942**	exon 11 Asp391Glu	16915134	0.125	CCTTAAGCAGCAACCACTCG	AGGAGACTGGGGGAGAAGAG	262

NVF= No variation found, NA= Not available/assay did not work properly, ‡= Location of exon and UTR sequence

oligonucleotides were designed, two of them allele specific and one common for both alleles. Depending on the position of the SNP in the amplicon allele specific primers were either forward or reverse and common primer correspondingly reverse or forward. In the 100-µl synthesis reaction 10 pmol of each primer (forward and reverse), 1X Klenow E.Coli Buffer, 0.4 mM dNTP, Klenow enzyme and 2 µl of 0.5 M EDTA was used. After synthesis 900 µl of dH_2O was added and cooled to 4°C. For using in Taqman reactions the artificial templates were diluted to 1/100 - 1/1 000 000, depending on the assay.

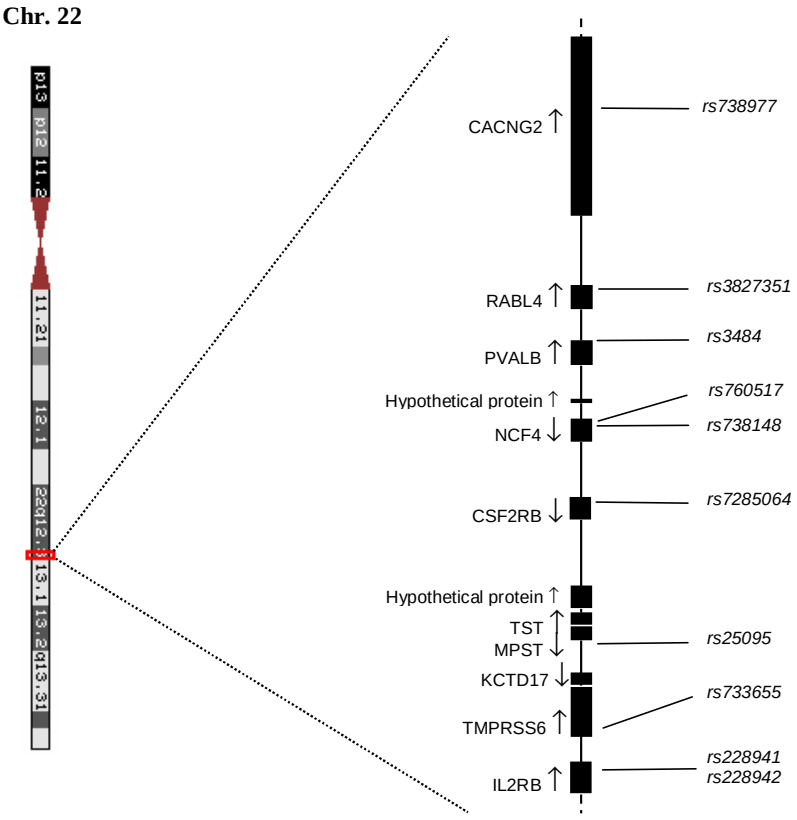


Figure 10. The selected SNPs cover a 541-kb region on chromosome 22q12-q13.

Table 25. Oligos used as primers in artificial template synthesis. Polymorphic base is in bold and underlined. Complementary sequence is indicated with an asterisk (*).

SNP	Oligo sequence 5' → 3'		
rs738977	**Forward T*** GGAGAGGATGCTTTTTACCAGTGGTTTACCGGCTGC**A**GGTATTTCAT	**Forward C*** GGAGAGGATGCTTTTTACCAGTGGTTTACCGGCTGC**G**GGTATTTCAT	**Reverse T/C** CACAGCATTAGCGGGAAAGGATGTGTCTAATGGATGAAATACC
rs3827351	**Forward C*** CGCTTGATAGAACTCAGAAATTAAAACCCACCCCAACCTCCAAAACA**G**GAGTCACCAA	**Forward A*** CGCTTGATAGAACTCAGAAATTAAAACCCACCCCAACCTCCAAAACA**T**GAGTCACCAA	**Reverse C/A** CCACTTCAACACCTCAACAGAGTTTAACCCGTAGCCCACTGGCCTTTGGTGACTC
rs3484	**Forward G*** CCTCTCTGCCCTGAACACCCAATCTCGGCCCCTCT**C**GCCACCCTCC	**Forward A*** CCTCTCTGCCCTGAACACCCAATCTCGGCCCCTCT**T**GCCACCCTCC	**Reverse G/A** AAACGAACTGAACAGAAATGCAGGAGGGTGGC
rs760517	**Forward C/T** GGCTGCCCTCCAAGGGTTCTTCCAACCTCGGCCTCCCCCAGGGCAGGCTCCAAAAC	**Reverse C*** TCAAGACACACTCGCACAGTTCACAGACTTTGGAGCCTGGCCTGGGGGA**G**GTTTTGGAGC	**Reverse T*** TCAAGACACACTCGCACAGTTCACAGACTTTGGAGCCTGGCCTGGGGGA**A**GTTTTGGAGC
rs738148	**Forward A** GGCCCTGGAGAGGAGCTAGTAGTGACCACTGGTTTAGCCAGTTT**A**GCAGTTTCCA	**Forward G** GGCCCTGGAGAGGAGCTAGTAGTGACCACTGGTTTAGCCAGTTT**G**GCAGTTTCCA	**Reverse A/G** GTAAGAGCCCTGAGTTCCTGAGATGGAGATCTATGATTAGGGTAAAATGGAAACTGC
rs7285064	**Forward C/T** TTGGGGATTTTCTGGCAGTGAGAAACTATGAGCTGGTCCTGGCCAGAAGCTCTGGACTCCAGCCTTCACCTACCCGACTCGGTGTCCCAGGAGCGCGCCTCACTCCACTC	**Reverse C** GGGTCAGGACCTCCCGCACCGGCTACAACGGGATCTGGAG**C**GAGTGGAGTG	**Reverse T** GGGTCAGGACCTCCCGCACCGGCTACAACGGGATCTGGAG**T**GAGTGGAGTG
rs25095	**Forward G*** AAGCGGGTGACTTAGTGTAATGATACTCAGCCTCACTTTCTATTGCAAT**C**GTTTCCCCAA	**Forward A*** AAGCGGGTGACTTAGTGTAATGATACTCAGCCTCACTTTCTATTGCAAT**T**GTTTCCCCAA	**Reverse G/A** CATTGCACAGGAGGTGCGAATACGTTAGGCTGGCTGGCAGATTCAGCGTTGGGGAAAC
rs733655	**Forward C/T** ACAGTGTGTGCTAACCACCTACTACATGGGGTACGCCAGTTAACCAAGACAGATGTGCCTCCCCT	**Reverse T*** TGCCAGAGCCACGCCTTTCTTACCCACCACTGTCAGCTTCAC**A**AGGGGAGGCA	**Reverse C*** TGCCAGAGCCACGCCTTTCTTACCCACCACTGTCAGCTTCAC**G**AGGGGAGGCA
rs228941	**Forward G/C** CCCTGACCTCTTCCCTGGGTTTTCTGCCCCAGCCTCCTCCTTCCCTCCCCTCCCC	**Reverse G*** AGCGTGGCCATATTTGGGTTTTGGAAAGCACGCTCAGGCTGCCCTGTGGA**C**GGGGAGGGGA	**Reverse C*** AGCGTGGCCATATTTGGGTTTTGGAAAGCACGCTCAGGCTGCCCTGTGGA**G**GGGGAGGGGA
rs228942	**Forward C*** ACCCTGTGGGTGCCCCGGCCACACCCTCATCAGG**G**TCTTCCTCTG	**Forward A*** ACCCTGTGGGTGCCCCGGCCACACCCTCATCAGG**T**TCTTCCTCTG	**Reverse C/A** AGGCCTGCCAGGTGTACTTTACTTACGACCCCTACTCAGAGGAAGA

4.6 Genotyping of SNPs for the refining of the breast cancer-associated region on 22q12-q13 (V)

For the ten selected SNPs forward and reverse primers and two fluorescent labelled probes were designed for 5' nuclease (Taqman) assays. Genotyping was carried out in 497 sporadic breast cancer cases and 458 matched controls according to assay manufacturer's instructions. The primer and probe sequences of the genotyped SNPs are shown in Table 26. In the 15-µl Taqman reactions we used 1x Taqman universal PCR master mix (Applied Biosystems), 900 nM forward and reverse primers (Applied Biosystems), 100, 200 or 400 nM each probe (Applied Biosystems) and 20 ng of genomic DNA. All assays were carried out in 96-well plates. Each plate contained 72 test samples, 8 negative controls with no DNA and 8 positive controls for each SNP allele. The following PCR conditions were used on an MJ Tetrad thermal cycler (MJ Research, Waltham, MA, USA) for Taqman® reactions: first 2 min at 50°C, then 10 min at 95°C followed by 39 cycles of 15 s at 95°C and 1 min at 60, 62 or 64°C. Samples were post-read on an AbiPrism 7700 Sequence Detector (Applied Biosystems) and were analysed using the Allelic Discrimination Sequence Detection Analysis 1.7 software (Applied Biosystems). Genotypes were manually assigned under dye components.

4.7 Allelic imbalance (AI) analysis (V)

4.7.1 Paraffin-embedded samples and extraction of DNA for AI analysis

Paraffin-embedded samples of breast cancer tissue from 45 of the 49 cases in study IV were used for the AI analysis in study V. An experienced pathologist microdissected the samples using a 24 G needle under a light microscope in carefully matched tumour areas containing at least 70 % tumour cells (Tuhkanen et al. 2004). For each specimen DNA was extracted from five 10 µm thick consecutive sections by the proteinase K-phenol-chloroform method according to standard protocols (Karjalainen et al. 2000).

Table 26. Primer and probe sequences of the genotyped SNPs.

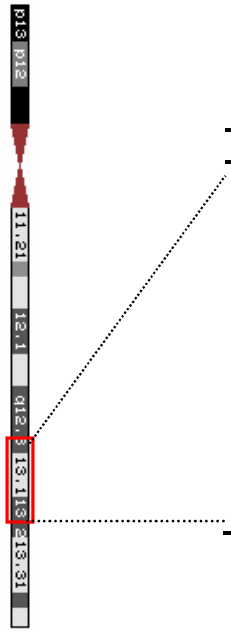
RefSNP ID	PCR primer sequence	C (nM)	Probe sequence [a, b]	C (nM)	Annealing temp.
rs738977	F: GAGGATGCTTTTTACCAGTGGTTT	900	A1: CGGCTGCGGGTATTTCATCCAT (v)[c]	200	62°C
	R: AGCATTAGCGGGAAAGGATGT	900	A2: CCGGCTGCAGGTATTTCATCCATT (f) [c]	200	62°C
rs3827351	F: TTGATAGAACTCAGAAATTAAAACCCAC	900	A1: CCAAAACATGAGTCACCAAAGGCCA (v) [c]	200	62°C
	R: CTTCAACACCTCAACAGAGTTTAACC	900	A2: CAAAACAGGAGTCACCAAAGGCCAGT (f) [c]	200	62°C
rs3484	F: CTCTGCCCTGAACACCCAA	900	A1: CTCGGCCCCTCTTGCCACC (v) [c]	200	62°C
	R: CGAACTGAACAGAAATGCAGGA	900	A2: CTCGGCCCCTCTCGCCAC (f) [c]	200	62°C
rs760517	F: TGCCCTCCAAGGGTTCTTC	900	A1: TCCAAAACCTCCCCCAGGCC (f)	400	60°C
	R: AGACACACTCGCACAGTTCACAG	900	A2: TCCAAAACTTCCCCCAGGCCA (v)	400	60°C
rs738148	F: CCTGGAGAGGAGCTAGTAGTGACC	900	A1: TTAGCCAGTTTGGCAGTTTCCATTTTACC (v)	400	64°C
	R: AGAGCCCTGAGTTCCTGAGATG	900	A2: TTTAGCCAGTTTAGCAGTTTCCATTTTACCCT (f)	400	64°C
rs1801117	F: GGGATTTTCTGGCAGTGAGAAA	900	A1: CTCACTCCACTCGCTCCAGATCCC (f) [c]	120	62°C
	R: TCAGGACCTCCCGCACC	900	A2: CTCACTCCACTCACTCCAGATCCCG (v) [c]	100	62°C
rs25095	F: CGGGTGACTTAGTGTAATGATACTCAG	900	A1: ACTTTCTATTGCAATTGTTTCCCCAACG (v) [c]	200	62°C
	R: TGCACAGGAGGTGCGAATAC	900	A2: TTCTATTGCAATCGTTTCCCCAACG (f) [c]	200	62°C
rs733655	F: GTGTGTGCTAACCACCTACTACATGG	900	A1: TGCCTCCCCTTGTGAAGCTGACA (v)	200	62°C
	R: CAGAGCCACGCCTTTCTTACC	900	A2: CCTCCCCTCGTGAAGCTGACAGTG (f)	200	62°C
rs228941	F: TGACCTCTTCCCTGGGTTTTCT	900	A1: CTCCCCTCCCCGTCCACAGG (f)	400	62°C
	R: GTGGCCATATTTGGGTTTTGG	900	A2: CCTCCCCCTCCACAGGGCA (v)	400	62°C
rs228942	F: CTGTGGGTGCCCCGG	900	A1: CACCCTCATCAGGGTCTTCCTCTGAG (f) [c]	100	62°C
	R: CCTGCCAGGTGTACTTTACTTACGA	900	A2: ACACCCTCATCAGGTTCTTCCTCTGAGTAG (v) [c]	100	62°C

[a] Probe label (v)= VIC, (f)= FAM. A1= allele 1 (major), A2= allele 2 (minor).
[b] Polymorphic base underlined.
[c] Complementary sequence.

4.7.2 Genotyping of microsatellites for AI analysis

For the AI studies 13 microsatellite markers from study IV were used (Figure 11.). These markers span an approximate 6.8 Mb on chromosome 22. Microsatellite repeats were PCR amplified from genomic (blood sample) DNA (in study IV) and from tumour DNA with fluorescent-labelled (FAM, HEX or TET) forward primers and an ordinary reverse primer using standard procedures (Sheffield et al. 1995). Fragment sizes were analysed using internal size standard, AbiPrism 310 Genetic Analyser (Applied Biosystems) and GeneScan 2.1 software (Applied Biosystems). Some of the markers were run multiplex. Intermarker distances and order were obtained from Human Genome Browser Gateway at the University of California at Santa Cruz (http://genome.ucsc.edu) and at http://www.sanger.ac.uk.

Chr. 22



Marker	Location
D22S277	34.596
D22S1142	34.598
D22S924	34.956
D22S283	35.075
D22S426	35.348
D22S1177	35.588
IL2RB	35.871
D22S445	35.890
D22S1156	36.706
D22S272	37.410
D22S284	38.641
D22S279	39.347
D22S270	41.378

Figure 11. Microsatellite markers used in the AI study. Location of each marker is shown in megabases (Mb) from the centromere.

4.7.3 Calculation of AI values

AI was calculated from the informative cases which were heterozygous in the normal tissue (blood) sample, using the peak heights from GeneScan analysis in formula AI = (T2 x N1) / (T1 x N2), where T is the tumour allele and N is the normal allele (1 and 2 representing the two alleles). An AI value greater than 1.67 or less than 0.60 was considered AI (Canzian et al. 1996). Samples with values from 0.6 to 0.7 and from 1.55 to 1.67 were re-amplified and reanalysed at this particular locus. Only when this second result was positive tumour was assessed to have AI. A locus with AI in more than 30 % of the cases was considered significant.

4.8 Statistical analyses (IV, V)

4.8.1 Microsatellite and SNP allele and genotype frequencies (IV, V)

Significance levels for comparisons of the microsatellite and SNP allele and genotype frequencies between cases and controls were computed using Fisher's exact test and Monte Carlo approximation implemented in SPSS. χ^2 and Fisher's exact tests were also performed to compare the frequency of the possible associated allele versus the frequency of all other alleles pooled together between cases and controls. *P*-values less than or equal to 0.05 were considered significant and *P*-values were unadjusted for multiple comparisons.

4.8.2 Hardy-Weinberg equilibrium (IV, V)

Deviation of the SNP and microsatellite genotype distributions from the Hardy-Weinberg equilibrium (HWE) was assessed using the standard χ^2 -test in Associate v2.33 or HWE softwares of the LINKUTIL software package (http://www.genemapping.cn/util.htm, Terwilliger and Ott 1994), analysing cases and controls combined as well as cases and controls separately. *P*-values less than 0.05 were considered as significant deviation from the HW equilibrium. *P*-values in HW equilibrium tests were not adjusted for multiple correction.

4.8.3 Breast cancer risk estimations (Odds ratios, OR) (IV, V)

Breast cancer-associated risks for the SNP genotypes and rare/common allele carriers and the associated alleles of the significant microsatellites were estimated as odds ratios (OR) with 95 % confidence intervals (CI) using crosstabulation in SPSS. Odds ratio estimation was considered reliable when the 95 % confidence interval did not intersect unity.

4.8.4 Haplotype estimation (IV, V)

In study IV the haplotype frequencies were computed using the 'haplo.score' software (Schaid et al. 2002). Haplo.score uses unphased genotype data and via the EM (Expectation Maximization) algorithm (Slatkin and Excoffier 1996) it estimates the haplotype frequencies. This software implements a global score test for haplotype frequency differences between the cases and controls, and provides haplotype-specific tests, which allow evaluation of all individual haplotypes when the global score test is significant. To improve the χ^2 approximation of the score test we used a threshold level of 0.005 and omitted haplotypes with frequency less than 0.5 %. Marker combinations where the global score test for differences in haplotype frequencies between cases and controls reached significance (at P=0.05 level), were investigated further by simulations (10 000). This involved simulating the case/control status of the individuals in the study and performing the test each time. The simulations were carried out using haplo.score.

In study V the haplotype frequencies for multiple SNP loci were estimated using the EM algorithm implemented in SNPAlyze v4.0 software (Dynacom, Chiba, Japan). SNPAlyze was also used for testing the association of the estimated haplotypes and breast cancer. SNPAlyze implements a global test for haplotype frequency differences between cases and controls and provides haplotype-specific tests which allow evaluation of all individual haplotypes. It also provides permutated (empirical) *P*-values for the global test and individual haplotypes. Permutation tests were done using FASTEHPLUS (Zhao and Sham 2002) and SNPAlyze. All permutated *P*-values were reached using 10 000 replicates. P-values $\leq$0.05 were considered significant.

4.8.5 Pairwise linkage disequilibrium (V)

Linkage disequilibrium (LD) between two SNPs was estimated by calculating the *D'*-values for all 45 possible pairs of the ten SNPs using the combined set of cases and controls (altogether 891-931 samples for each SNP) (Devlin and Risch 1995).

4.8.6 Power estimations (V)

Power estimations for the SNP association studies were calculated using the Genetic Power Calculator at http://pngu.mgh.harvard.edu/~purcell/gpc/, case-control for discrete traits (Purcell et al. 2003). In these estimations we used α=0.05 and breast cancer prevalence of 0.8 % (http://www.cancerregistry.fi/). *D'* was set at 1 (assuming complete linkage disequilibrium between marker allele and disease-associated allele) and the allele frequencies were assumed equal for the risk SNP and the marker SNP. Also the risks were assumed equal (1.5 or 2) for the homozygous and heterozygous high-risk allele carrying genotypes.

4.9 Ethical aspects

All study participants gave their informed written consent. The mutation screening study was approved by the joint ethics committee of Kuopio University and Kuopio University Hospital and appropriate permission from the Ministry of Social Affairs and Health in Finland was obtained. The Kuopio Breast Cancer Project was approved by the joint ethics committee of Kuopio University and Kuopio University Hospital.

5. RESULTS

5.1 *BRCA1* and *BRCA2* mutations in breast/ovarian cancer families (I-III)

Altogether 89 individuals in 36 breast and ovarian cancer families were studied and five different *BRCA1/BRCA2* gene mutations were found; two in *BRCA1* gene and three in *BRCA2* (Table 27). In addition to the truncating mutations three polymorphisms were detected in the *BRCA1* gene and seven in *BRCA2* gene (Table 28).

Table 27. *BRCA1* and *BRCA2* gene mutations found.

Mutation	Family	Index case*	No. of brca in family†	No. of ovca in family§	Other cancers in family‡
BRCA1:					
ex 12 4216-2ntA→G	BR01	brca (36)	1 (50)	-	endometrial (50)
ex 12 4216-2ntA→G	OV01	ovca (46)	2 (51, 43)	1 (38)	na
ex 20 5730 C→T	PS04	-	2 (32, 52)	-	melanoma (77), liver (43), thyroid (60-70), oesophageal (65), lymph node (29) endometrial (73)
BRCA2:					
ex 9 999del5	BR17	brca (34, 45)	4 (25, 40 bilat., 43, 58)	-	melanoma (35)
ex 9 999del5	OV30	ovca (62)	2 (27, na)	2 (na, na)	gastric (na), lip (na), lymphoma (4), pancreas (na)
ex 11 4088insA	BR09	brca (74, 77)	2 (86, 46)	3 (61, 58, 58 bilat.)	rectal (57), prostate (80)
ex 11 6503delTT	OV03	ovca (51)	5 (37, na, na, na, na)	1 (na)	brain (na)

*Carcinoma and age at diagnosis of the index patient
†Number of breast cancer cases in the family, index case not included, age at diagnosis in parenthesis.
§Number of ovarian cancer cases in the family, index case not included, age at diagnosis in parenthesis.
‡Other cancers in family, age at diagnosis in parenthesis.
na=not available

5.1.1 *BRCA1* mutations found (I, III)

The detected *BRCA1* gene mutations were 4216-2nt A→G in exon 12, and 5370 C→T in exon 20. The 5370 C→T mutation in exon 20 was found in sequencing analysis and

exon 12 4216-2nt A→G mutation was detected in heteroduplex analysis and confirmed by RFLP and sequencing from genomic DNA and cDNA.

Table 28. Polymorphisms found in the *BRCA1* and *BRCA2* genes.

Gene/ Exon/Intron	Alteration*	Effect	rs Number †	Observed in Cases	Observed in Controls
***BRCA1*:**					
Intron 7	-34ntT→C	IVS7 -34T⟶C	-	1/24	na
Exon 13	T4427C	Ser1436Ser (S1436S)	rs1060915	12/24	na
Exon 16	A4956G	Ser1613Gly (S1613G)	rs1799966	11/24	
***BRCA2*:**					
5'UTR	203G→A	5'UTR -26nt G→A	rs1799943	14/33	6/31 ‡
Intron 8	+56ntC→T	IVS8 +56C⟶T	rs2126042	7/33	9/31 ‡
Exon 10	A1093C	Asn289His	rs766173	1/29	na
Exon 10	A1342C	Asn372His	rs144848	17/30	na
Exon 10	A1593G	Ser455Ser	rs1801439	1/34	na
Intron 14B	+53ntC→T	IVS14 +53C⟶T	rs11147489	1/33	0/31 ‡
Intron 24	-16ntT→C	IVS24 -16T⟶C	rs11571818	1/33	1/31 ‡

* nt corresponding to mRNA sequence GenBank U43746; † RefSNP number, obtained from the NCBI dbSNP at http://www.ncbi.nih.gov/SNP/. ‡ Anonymous population controls were used. na= not analysed

5.1.1.1 *BRCA1* exon 12 4216-2nt A→G (III)

The *BRCA1* exon 12 4216-2nt A→G mutation was found in 3/8 and 3/12 individuals tested in two families. This mutation substitutes the adenine (A) nucleotide at position 4216-2nt to guanine (G) and disrupts the splice acceptor AG dinucleotide. The substituted guanine creates a new splice acceptor AG with an adenine at position 4216-3nt causing splicing error which leads to the inclusion of an extra nucleotide G in the mRNA. Aberrant splicing causes a frame-shift and changes the codon GGU for Glysine at position 1366 to GGG and codon GAA for Glutamine at 1367 to translation termination codon UGA (Figure 12). This leads to a truncated BRCA1 protein of 1366 amino acid instead of the 1863-amino-acid wild-type protein.

In family A (BR01) the 4216-2nt A→G mutation was found in one breast cancer patient (female) and in two healthy individuals (male and female). Other five tested family members (male and female) were non-carriers. In family OV01 one breast cancer patient, one ovarian cancer patient and one healthy male were 4216-2nt A→G mutation

carriers. Nine other tested family members were non-carriers, one of them being affected with breast cancer at the age of 51 years.

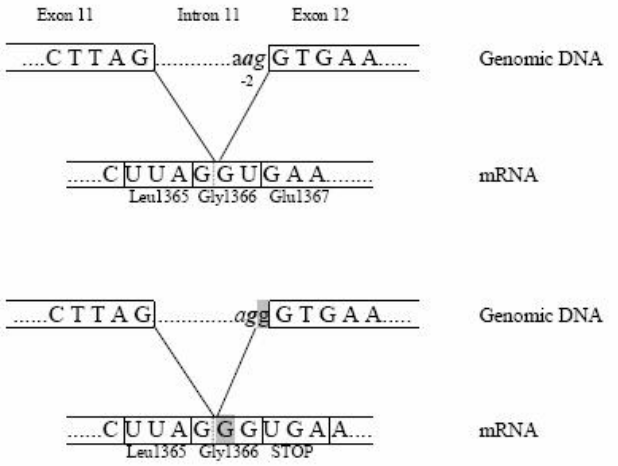


Figure 12. The 4612-2nt A to G splice-site mutation in *BRCA1* gene leads to aberrant splicing and truncated protein. Wild-type sequence is shown in the upper part and mutated sequence in the lower part. The splice acceptor *ag* dinucleotide is shown in italics.

5.1.1.2 *BRCA1* exon 20 5370 C→T (I)

The 5370 C→T mutation in exon 20 was found in one family (PS04). This mutation changes the codon for Arginine (AGA) at position 1751 to a translation termination codon UGA. This leads to a truncated BRCA1 protein of 1751 amino acid. The mutation was found in the only individual (female melanoma patient) tested in the family.

5.1.2 *BRCA2* mutations found (I, II)

Three mutations were detected in the *BRCA2* gene; 999del5 in exon 9, 4088insA in exon 11 and 6503delTT in exon 11. The 4088insA and 6503delTT mutations were detected in PTT analysis and confirmed by sequencing. Exon 9 999del5 was detected in heteroduplex analysis and confirmed by sequencing.

5.1.2.1 *BRCA2* exon 9 999del5 (I)

The exon 9 999del5 mutation was found in two families. This mutation is a five base-pair deletion TCAAA, starting at nucleotide 999 in codon 257. The deletion causes a frameshift leading to early translation termination in codon 273 and a truncated protein of 273 amino acids instead of the 3418-amino-acid wild-type protein.

In family BR30 the mutation was found in a breast cancer patient who was the only individual whom a sample was available for testing.

In family OV30 the 999del5 mutation was found in three of eleven tested family members. One of the mutation carriers had ovarian cancer and the other two were healthy females.

5.1.2.2 *BRCA2* exon 11 4088insA (II)

The 4088insA mutation was found in one family (BR09). The insertion of an adenine in a chain of 7 adenines at nucleotides 4081-4087 causes a frameshift leading to the change of Asparagine (AAT) at codon 1287 to Lysine (AAA), and the Asparagine (AAT) at 1288 to a stop codon UAA. This leads to premature translation termination at 1288 and a truncated protein of 1288 amino acids (Figure 13). In the family BR09 the mutation was found in 6/11 tested family members. One male carrying the mutation is healthy. One male carrier is affected with prostate cancer and four female carriers are affected with bilateral breast cancer, bilateral ovarian cancer, breast, ovarian and rectal cancer, and one with breast and ovarian cancer. Other five tested family members were non-carriers.

5.1.2.3 *BRCA2* exon 11 6503delTT (I)

The third *BRCA2* gene mutation detected was 6503delTT in exon 11. This mutation was found in one family (OV03). The deletion of nucleotides 6503 and 6504 causes a frameshift starting at codon 2092 and creates a stop codon at 2098 leading to a truncated protein of 2098 amino acids. In the family OV03 four 6504delTT mutation carriers were detected, three of them healthy (one male and two females) and one affected with ovarian cancer. The three other tested family members were non-carriers.

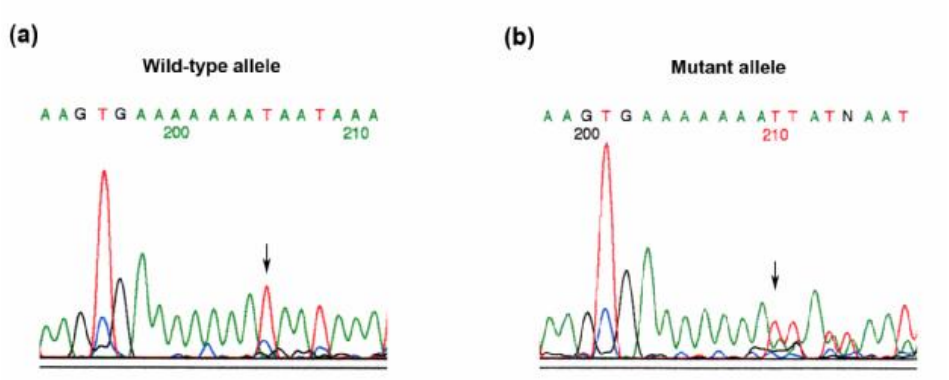


Figure 13. Sequencing of the *BRCA2* 4088insA mutation. (**a**) Wild-type sequence in the region of the mutation. The arrow indicates the position of the adenine insertion. (**b**) Sequence of the 4088insA mutated allele. The arrow indicates the inserted adenine. The frame-shift resulting from the adenine insertion is visible as tangled sequence.

5.1.3 Haplotype analysis (I, II)

Three-marker haplotypes consisting of alleles at markers *D13S260*, *D13S1701* and *D13S267*, spanning 1.83 Mb (3.2 cM), were manually constructed for the three *BRCA2* exon 9 999del5 mutation carriers and 8 non-carriers in family OV30. The mutation carriers shared haplotype 166 - 304 - 258. This haplotype was not detected in any of the non-carriers. In family BR17 a DNA sample was available only from the mutation carrier and her haplotype was 168/174 - 292/304 - 252/256. It is likely that the allele 304 of the closest marker to the 999del5 mutation, *D13S1701*, is shared by all mutation carriers in families OV30 and BR17.

In family OV03 with *BRCA2* exon 11 6503delTT mutation the haplotype using markers *D13S260*, *D13S1701* and *D13S267* was constructed for two mutation carriers and two non-carriers. The 6503delTT mutation associated haplotype was 170 - 292 - 244/256 and it was not detected among the non-carriers.

Two-marker haplotype was constructed for six mutation carriers and five non-carriers in family BR09 with the *BRCA2* 4088insA mutation using markers *D13S260* and *D13S267*. All mutation carriers shared the haplotype 166 - 250 and it was not detected in the non-carriers.

Haplotypes in families BR01, OV01 and PS04 carrying *BRCA1* 4216-2ntA→G and 5370 C→T mutations, have been assessed as part of the collaborative analysis of haplotypes and founder effects of *BRCA1* and *BRCA2* families in Finland (Sarantaus et al. 2000).

5.2 Autosome-wide scan for linkage disequilibrium-based association with breast cancer (IV)

5.2.1 Allelic association

In the first-stage scan 366 microsatellite markers were genotyped on 22 chromosomes in 49 breast cancer cases and 50 controls from Eastern Finnish population. In 16 chromosomes altogether 21 markers showed significant difference in allele frequencies when compared between breast cancer cases and controls, with *P*-values 0.003-0.049 in Fisher's exact test, indicating that these markers are in LD with breast cancer (Study IV, Table 1). In addition, two markers associated with breast cancer with borderline significance, with *P*-values 0.054 and 0.057 (Study IV, Table 1). More than one marker on same chromosome was detected to be in LD with breast cancer on 5 chromosomes, but none of these markers were adjacent. On six chromosomes no markers were detected to be in LD with breast cancer. For the second-stage scan 23 loci (21 with the most significant *P*-values and 2 near significant) on 16 chromosomes were selected and altogether 69 additional flanking markers were analysed. Four of the 69 analysed markers showed significant LD with breast cancer with *P*-values 0.013-0.046 (Study IV, Table 1). The breast cancer association was further analysed by single-allele tests for 25 of the 27 significant markers where the difference between cases and controls in the count of the tested allele was ≥ 10. Thirty-nine alleles of 25 markers were tested and 28 alleles showed significant association, two showed borderline significance and 8 were not significant (Study IV, Table 1). The odds ratios indicated 9 alleles possibly being protective (OR 0.013-0.47) and 21 alleles associating with increased breast cancer risk (OR 1.84-12.86) (Study IV, Table 1).

5.2.2 Genotype association and Hardy-Weinberg equilibrium (HWE)

Among all analysed markers (366+69) only six markers showed significant difference in genotype frequencies between cases and controls. These were *D3S3053*, *D7S1818*, *D19S178*, *D19S254*, *D22S1177* and *D22S445*, with *P*-values 0.041, 0.014, 0.048, 0,007, 0.040 and 0.010, respectively. In the HWE analysis the genotypes of controls deviated significantly from equilibrium at one marker, *D1S179*, and slightly at *D22S426* (Study IV, Table 1). The cases deviated also at *D1S179* and at *D17S809* with *P*-values ~0 and 0.011, respectively. In the combined analysis of cases and controls together deviation from HWE was detected with markers *D1S179*, *D17S809* and *D22S426* with *P*-values ~0, 0.013 and 0.006, respectively. The substantial deviation at marker *D1S179* in all three tests is probably due to the large number of observed alleles, 15, at this marker.

5.2.3 Haplotypes

In the haplotype analysis 76 different marker combinations were tested for two-, three and four-marker haplotypes. Four marker combinations showed significant difference in haplotype frequencies between cases and controls with global test score ≤ 0.05 and simulated *P*-values of 0.003-0.021 (Study IV, Table 2). In addition, three other showed borderline significance with global scores 0.077, 0.095 and 0.068 (Study IV, Table Table 2). With the four significant marker combinations the number of observed haplotypes with frequency of ≥ 0.5 % varied from 22 to 45. Among these individual haplotypes four were found more frequently in cases and seven were more frequent in controls (*P*-values 0.007-0.038, simulated *P*-values 0.004-0.030; Study IV, Table 2). The simulations and haplotype frequency estimations for cases and controls separately were not done for the three marker combinations with borderline significance.

The results from allelic association and haplotype analysis suggest that in the Eastern Finnish population breast cancer associated regions locate on chromosomes 3p26, 11q23 and 22q12-q13.

5.3 Further refinement of chromosome 22q12-q13 breast cancer-associated region (V)

5.3.1 SNP allelic and genotype association

Ten SNPs were genotyped in the 550-kb breast cancer-associated region on chromosome 22q12-13, detected in the autosome-wide scan in study IV. Breast cancer association of the SNPs were tested by comparing the allele and genotype frequencies between 497 breast cancer cases and 458 controls using the entire sample and a subsample that included the cases born in the province of Northern Savo (n=280) and their age and long-term area-of-residence matched controls (n=257). In the entire sample no significant difference were detected in allele frequencies between cases and controls with any of the ten SNPs but a borderline significance was detected for the rare allele carriers (genotypes TC and CC) of rs733655 with an OR of 1.28 (95% CI=0.99-1.65, Table 29). In the subsample two SNPs associated with breast cancer, rs728506 with *P*-value 0.033, and rs733655 with *P* of 0.009 (Table 29). In the subsample also the rs733655 rare allele carriers (genotypes TC and CC) had a significant risk of breast cancer compared to the homozygous common allele carriers (TT) with an OR of 1.90 (95% CI=1.34-2.69). The genotype frequencies differed significantly with rs733655 in the entire sample (P=0.044) and the heterozygous genotype TC was the risk-associated genotype with OR 1.39 (95% CI=1.06-1.83, Study V, Table 2). Breast cancer association with rs733655 was detected also in the subsample where the genotype frequencies differed between cases and controls significantly with *P* of 0.0003 and the TC genotype associated with breast cancer risk with an OR of 2.11 (95% CI=1.46-3.05, Study V, Table 3). In the subsample a protective effect associated with rs7285064 heterozygous genotype CT with OR 0.62 (95% CI=0.41-0.96, Study V, Table 3).

5.3.2 Hardy-Weinberg equilibrium

The deviation of the genotype frequencies from the Hardy-Weinberg equilibrium was tested separately for cases and controls in each sample (entire and subsample). In the controls the rs733655 genotypes were slightly off from HWE in the entire sample and more significantly in the subsample. A borderline deviation was detected among cases

Table 29. SNP allele frequencies, allele frequency comparisons and allele specific risks.

				Allele Frequency				Rare allele carrier risk				Common allele carrier risk			
	*P**			Entire sample†		Subsample§		Entire sample†		Subsample§		Entire sample†		Subsample§	
SNP	Entire sample†	Subsample§	Allele	Cases	Controls	Cases	Controls	OR	CI	OR	CI	OR	CI	OR	CI
rs738977	0.958	0.296	C	0.834	0.835	0.864	0.84					0.93	0.44-1.95	0.75	0.26-2.13
			T	0.166	0.165	0.136	0.16	1.00	0.75-1.32	0.75	0.51-1.11				
rs382735	0.581	0.837	A	0.718	0.730	0.710	0.717					0.87	0.54-1.41	0.81	0.44-1.47
			C	0.282	0.270	0.290	0.283	1.05	0.81-1.37	0.99	0.70-1.39				
rs3484	0.118	0.620	A	0.559	0.532	0.552	0.536					1.13	0.82-1.55	1.07	0.71-1.63
			G	0.441	0.477	0.448	0.464	0.78	0.58-1.03	0.91	0.62-1.32				
rs760517	0.493	0.894	C	0.687	0.672	0.681	0.677					1.00	0.65-1.54	1.01	0.59-1.73
			T	0.313	0.328	0.319	0.323	0.89	0.68-1.15	0.97	0.69-1.37				
rs738148	0.501	0.170	G	0.729	0.715	0.733	0.694					1.29	0.80-2.08	1.41	0.77-2.57
			A	0.271	0.285	0.267	0.306	0.96	0.74-1.25	0.82	0.58-1.15				
rs728506	0.470	**0.033**	C	0.883	0.872	0.904	0.861					1.33	0.44-3.99	1.52	0.34-6.84
			T	0.117	0.128	0.096	0.139	0.90	0.66-1.23	0.62	0.41-0.95				
rs25095	0.202	0.260	A	0.541	0.511	0.558	0.521					1.23	0.90-1.69	1.40	0.91-2.14
			G	0.459	0.489	0.442	0.479	0.88	0.66-1.18	0.91	0.62-1.34				
rs733655	0.254	**0.009**	T	0.721	0.744	0.687	0.76					1.22	0.75-1.98	1.18	0.63-2.20
			C	0.279	0.256	0.313	0.24	1.28	0.99-1.65	**1.90**	**1.34-2.69**				
rs228941	0.292	0.172	G	0.706	0.728	0.698	0.737					0.92	0.57-1.48	0.54	0.26-1.10
			C	0.294	0.272	0.302	0.263	1.16	0.90-1.50	1.17	0.83-1.65				
rs228942	0.163	0.315	C	0.796	0.821	0.791	0.817					0.88	0.45-1.71	0.88	0.34-2.27
			A	0.204	0.179	0.209	0.183	1.15	0.88-1.50	1.23	0.86-1.75				

**P*-value from chisquared Fisher's exact test for the difference in allele frequencies cases vs. controls.
† 497 breast cancer cases and 458 controls.
§ 280 breast cancer cases and 257 controls.

in the subsample in rs738977 and rs733655 genotypes with *P*-values 0.052 and 0.082, respectively. (Study V, Tables 2 and 3).

5.3.3 Pairwise linkage disequilibrium

Linkage disequilibrium between SNPs was estimated in the entire sample by calculating pairwise *D'* values for all 45 possible SNP pairs. In six of the 45 pairs a D'≥0.5 were observed (Table 30). *D'* was detected to decline as distance between SNPs increased, as was expected. The highest *D'*, 0.99, was detected between rs228941 and rs228942 which are in the same gene (*IL2RB*) and only 898 kb apart. Moderate LD with *D'* 0.49-0.61 was observed between SNPs that are up to 236 kb apart.

Table 30. *D'* values for pairwise linkage disequilibrium in the entire sample (cases and and controls combined). The distance between a SNP pair is shown in kilobases (kb) in the upper half and *D'* value in the lower half.

	rs738977	**rs3827351**	**rs3484**	**rs760517**	**rs738148**	**rs7285064**	**rs25095**	**rs733655**	**rs228941**	**rs228942**
rs738977		148.3	187.9	250.1	261.6	321.1	425.1	486.1	514.8	515.7
rs3827351	0.56		39.6	101.7	113.3	172.8	276.8	337.8	366.5	367.4
rs3484	0.05	0.59		62.1	73.6	133.1	237.2	298.2	326.9	327.7
rs760517	0.08	0.26	0.17		11.5	71.0	175.0	236.1	264.7	265.6
rs738148	0.12	0.04	0.10	0.57		59.5	163.5	224.5	253.2	254.1
rs7285064	0.14	0.06	0.08	0.16	0.18		104.0	165.1	193.7	194.6
rs25095	0.04	0.08	0.12	0.15	0.06	0.25		61.0	89.7	90.6
rs733655	0.13	0.28	0.26	0.04	0.28	0.09	0.49		28.7	29.6
rs228941	0.02	0.03	0.13	0.10	0.24	0.19	0.24	0.05		0.9
rs228942	0.23	0.08	0.02	0.07	0.24	0.61	0.01	0.02	0.99	

5.3.4 Haplotypes

Two-marker haplotypes were estimated for the adjacent SNPs that were in LD with $D' \geq 0.5$. Altogether five pairs were tested separately in the entire sample and subsample. The global chi-squared *P* value and empirical *P* were significant for only one marker pair in the subsample, rs25095-rs733655. An individual haplotype AC of this SNP pair is significantly more frequent in cases with empirical *P* of 0.003 (Study V, Table 5). In the entire sample this haplotype has a borderline significance with empirical *P* 0.073, but non-significant global *P*. Other haplotypes were not significant.

5.3.5 Allelic imbalance analysis

In 37/45 (82%) of the studied breast tumours AI was detected at one or more of the 13 microsatellite marker loci analysed on chromosome 22q12-q13. Five microsatellite loci, *D22S1142*, *D22S924*, *D22S1177*, *D22S445* and *D22S279*, showed significant AI (Table 31).

Table 31. Allelic imbalance detected on chromosome 22q12-q13.

		AI	
Marker	**Location (Mb)**	**n***	**%†**
D22S277	34.596	5/35	14
D22S1142	34.598	12/36	**33**
D22S924	34.956	15/33	**45**
D22S283	35.075	11/40	28
D22S426	35.348	2/12	17
D22S1177	35.588	12/22	**55**
IL2RB	35.871	10/40	25
D22S445	35.890	9/26	**35**
D22S1156	36.706	2/25	13
D22S272	37.410	8/32	25
D22S284	38.641	9/35	26
D22S279	39.347	7/18	**39**
D22S270	41.378	1/30	3

* Number of tumours showing AI/Total number of informative tumours.
† AI percentage.

6. DISCUSSION

6.1 High-risk susceptibility genes in Eastern Finnish breast and ovarian cancer families (I-III)

6.1.1 Eastern Finnish *BRCA1* and *BRCA2* mutation frequency is not different from other parts of Finland (I)

Five different *BRCA1* and *BRCA2* gene mutations were found in the Eastern Finnish population; 4216-2nt A→G in exon 12, and 5370 C→T in exon 20 of *BRCA1* gene, and 999del5 in exon 9, 4088insA in exon 11 and 6503delTT in exon 11 of *BRCA2*. These mutations account for 19.4 % of the 36 families studied. *BRCA1* mutations were detected in 8.3 % (3/36) and *BRCA2* mutations in 11.1 % (4/36) of the families. In the previous studies conducted in Northern or Southern Finnish families the corresponding figures were 7 % and 10 % for *BRCA1*, and 6 % and 11% for *BRCA2* (Huusko et al. 1998, Vehmanen et al. 1997a). Thus, the frequencies that we observed do not differ significantly from those previously reported in Finland.

Some mutations in the present study may have remained undetected since the sensitivity of mutation detection methods is not complete. It is also possible to miss some mutations due to phenocopies as in some families DNA from only one breast or ovarian cancer case was available for mutation screening. However, missing many mutations is unlikely because 17 previously detected Finnish *BRCA1* and *BRCA2* mutations were tested, and the mutation frequencies are similar to those previously reported in Finnish breast/ovarian cancer families. Also, large genomic rearrangements involving *BRCA* genes have not been found in the Finnish population (Lahti-Domenici et al. 2001, Laurila et al. 2005, Karhu et al. 2006).

In addition to truncating mutations three polymorphisms were detected in the *BRCA1* gene and seven in *BRCA2* gene. All ten polymorphisms are known and have been found in other populations. *BRCA2* Asn372His has been associated with an increased risk of breast cancer although in a very large collaborative sample set the association was not confirmed (Healey et al. 2000a, Breast Cancer Association Consortium 2006). In Kuopio Breast Cancer Project sample material the association was not significant

(Healey et al. 2000a). Some evidence was however reported of the 372His homozygotes in the youngest age group having an increased risk of breast cancer (Breast Cancer Association Consortium 2006). Increased risk of ovarian cancer has also been detected among Asn372His carriers (Auranen et al. 2003). None of the other nine polymorphisms has been reported to associate with breast/ovarian cancer risk. Thus, it is unlikely that these polymorphisms contribute significantly to risk of familial breast cancer in our sample material.

In general, the mutations in the present study were found in families with several cancer cases. However, in many families with breast and ovarian cancer a mutation was not found, and two mutations were found in families with only two cases of breast cancer. The *BRCA1* 5370 C→T mutation was found in a family in which only two cases of breast cancer (second-degree relatives) were known, but additionally many cases of other cancers. The mutation in this family was found from a patient with melanoma, and DNA from the breast cancer cases was not available for testing. The *BRCA1* 4216-2nt A→G mutation was also detected in a family where the index case had breast cancer at the age of 36 and her mother had endometrial and breast cancers but no other cancer cases were known in the family. This demonstrates that very strict criteria, i.e. three first-degree relatives with breast/ovarian cancer, for *BRCA1/2* mutation testing are not applicable in all cases.

In the present study *BRCA* mutations were not detected in any of the six families with ovarian cancer only, which is consistent with the previous findings. In a study of 233 unselected Finnish ovarian cancer patients *BRCA1/2* mutations were detected in 5.6 % of the patients (Sarantaus et al. 2001b). Mutations were present in 13 families and only two of them did not have a history of breast cancer in the family, in addition to ovarian cancer (Sarantaus et al. 2001b). Even though the number of families with ovarian cancer only is low in the present study, it supports the previous conclusion that ovarian cancer patients who have also been diagnosed with breast cancer or have family history of breast or breast/ovarian cancer could benefit from referral to genetic counselling and *BRCA* mutation testing (Sarantaus et al. 2001b).

6.1.2 Limited *BRCA1* and *BRCA2* mutation spectrum in Eastern Finland (I)

Although the *BRCA1* and *BRCA2* mutation frequencies observed in the present study correspond to those previously found in Finland, the mutation spectrum, however, is different. We detected only two of the 15 previously reported *BRCA1* and two of the 11 reported *BRCA2* mutations (Vehmanen et al. 1997a, Vehmanen et al. 1997b, Roth et al. 1998, Huusko et al.1998, Sarantaus et al. 2000, Syrjäkoski et al. 2000, Sarantaus et al. 2001a, Sarantaus et al. 2001b, Pääkkönen et al. 2001, Eerola et al. 2001, Vahteristo et al. 2001a, Syrjäkoski et al. 2004a). The Eastern Finnish (Northern Savo) mutation spectrum resembles that of the Northern part of the country where only a few *BRCA1/2* mutations have been found (Huusko et al. 1998). Two of the four recurrent mutations that we detected represent the most frequently observed *BRCA1/2* gene founder mutations in Finland, 4216-2nt A→G in *BRCA1* and 999del5 in *BRCA2*. The other two, *BRCA1* 5370 C→T and *BRCA2* 6503delTT, have been less frequently detected in Finland and have been observed in other countries too. In addition to *BRCA1* 4216-2nt A→G and *BRCA2* 999del5, the three other most frequent Finnish mutations in each gene were not detected in the Eastern Finnish families (*BRCA1* 3604delA, 3744delT/ 3745delT and 4446C→T, and *BRCA2* 7708C→T, 8555T→G and 9346-2ntA→T). Additionally, we observed the 4088insA mutation in *BRCA2* exon 11. In Finland *BRCA2* 4088insA mutation is unique to the family described in study II. It has been reported in the BIC database to have been found in one Italian ovarian cancer patient but not anywhere else.

However, it should be kept in mind that the 36 families in the present study cover only a portion of the families with hereditary breast and ovarian cancer in the Northern Savo region, and are a portion diagnosed in the 1990's. It is possible that if the study material was larger some of the most prevalent founder mutations would be found, especially *BRCA2* 7708C→T and 9346-2ntA→T, which have been described in Finnish families originating from Northern Savo (Sarantaus et al. 2000).

6.1.3 Geography and haplotypes in the *BRCA* mutation-carrier families (I, II)

The *BRCA1* gene 4216-2nt A→G mutation was detected in two families, which are from the Northern part of Central Finland and in Northern Savo region (Figure 14). In the collaborative analysis the origins of the Finnish families with 4216-2nt A→G mutation were observed to cluster in Central Ostrobothnia and further in Central Finland (Sarantaus et al. 2000).

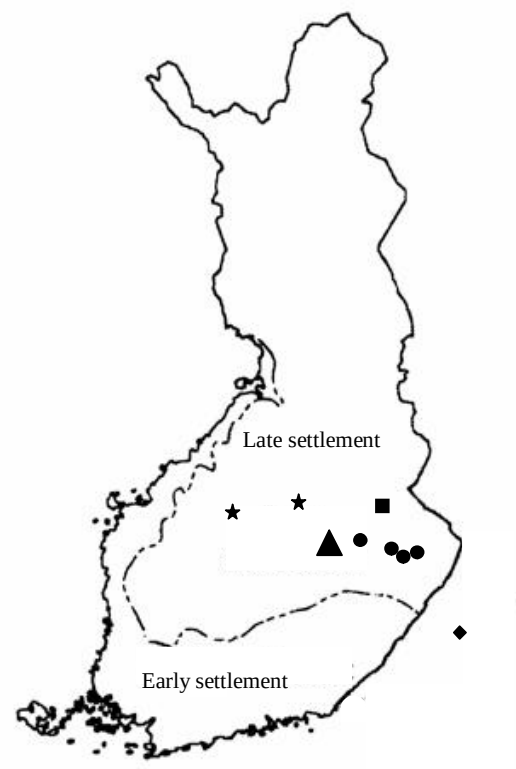


Figure 14. Birth places of the oldest generation available in five *BRCA1/2* mutation families. Black circles represent the birth places of *BRCA2* 999del5 mutation family (grandparents of index case in family OV30), black square *BRCA2* 6503delTT (father of index case in family OV03), star *BRCA1* 4216-2nt A→G mutation families (index cases of families BR01 and OV01), diamond *BRCA2* 4088insA (index case of family BR09). Location of Kuopio is shown with a triangle.

The origin of the *BRCA1* 5370 C→T mutation families in Finland seems to be in the most Eastern parts, or in Russia (Sarantaus et al. 2000). The birthplace of the oldest generation known (father of the index case) in the family with *BRCA2* 6503delTT

mutation is also from Eastern Finland, close to Kuopio (Figure 14). The single family carrying *BRCA2* 4088insA mutation originates from Karelia (Figure 14).

The *BRCA2* 999del5 mutation was also detected in two families. In one of the families all four grandparents of the index case could be traced to have been born in a small region in Eastern Finland, communities in Northern Savo and Northern Karelia (Figure 14). In the other family with 999del5 mutation the birth places were not available. A haplotype analysis in Finnish and Icelandic breast cancer families with *BRCA2* 999del5 mutation revealed two distinct haplotypes; a rare one that shared a core-haplotype with the Icelandic haplotype, and another one more frequent in Finland (Barkardottir et al. 2001). Families with this more frequent haplotype originated from two regions in Finland, the other of them in Eastern Finland near Kuopio where the family in the present study also originates.

Our results support the idea of the birth of population islets in Finland during resettlement of the Eastern parts of Finland in the 16th century. Only two of the eight most common Finnish founder mutations were detected in our material. In addition, the novel mutation *BRCA2* 4088insA has not been found in Southern or Northern Finland. Our study agrees with the suggestion that there are only a limited number of *BRCA1*/*BRCA2* mutations in the Finnish population and the majority of them have been found. The relatively low total number of known *BRCA1*/*BRCA2* mutations in Finland, including the ones reported in this study, suggests that other possibly moderate risk inducing gene mutations may exist in the Finnish population. Indeed, recently a founder mutation in *PALB2* gene has been found in Finnish breast cancer families, including a family from Kuopio region (Erkko et al. 2007). The knowledge of founder mutations representative of certain geographical regions facilitates the laborious mutation screening of large *BRCA1* and *BRCA2* genes.

6.1.4 A novel Eastern Finnish *BRCA2* mutation and favourable prognosis (II)

In the present study we have identified a rare 4088insA mutation in the *BRCA2* gene. In addition to the Eastern Finnish family in the present study it has not been detected in other families in Finland. The mutation is associated with high penetrance: all four

female mutation carriers have presented with breast/ovarian cancer and all of them were either bilateral breast cancer or ovarian cancer, or breast and ovarian cancer in the same patient. One of the two male mutation carriers has prostate cancer. The 4088insA mutation seems to be of an auspicious nature as despite of even an advanced stage and poor prognosis assessed by classical factors all affected family members had an excellent response to treatment and a better than expected clinical course. Still, the mutation is persistent as breast cancer occurred in a patient even 25 years after the first (ovarian) cancer. On the other hand, the patient diagnosed with bilateral ovarian cancer of the size of two grape fruits and metastatic spreads, was also cured and is still disease free and alive after nearly 26 years.

An interesting question is whether the mutation itself is associated with good prognosis/outcome or is there another gene that modifies the outcome of the mutation-associated cancers in the family. Also, as *BRCA2* gene functions in double-stranded DNA break (DSDB) repair, cells deficient in functional BRCA2 are more sensitive to agents that cause DSDB (Patel et al. 1998, Sharan et al. 1997, Abbott et al. 1998). Therefore certain chemotherapeutic drugs and radiation may be especially efficient in *BRCA2*-deficient cancer patients but on the other hand the radiation therapy in mutation carriers may initiate tumorigenesis by causing DSDB that are not subsequently repaired. Indeed, some evidence for *BRCA2*-deficient cancer cells being more sensitive to ionizing radiation, mitoxantrone, amsacrine and etoposide have been presented (Sharan et al. 1997). Also *BRCA1/2*-related breast cancer cases had a better clinical response rate to neoadjuvant chemotherapy than non-carriers (Chappuis et al. 2002). In the family described here the patient with bilateral breast cancer did not receive radiation therapy for the first breast cancer and patient who received radiation therapy for breast cancer had subsequent rectal and ovarian cancers which are unlikely due to scatter dose of radiation to breast. The third breast cancer patient in the family has also received radiation therapy, but only recently, and has not developed bilateral breast cancer. Better response to platinum chemotherapy has been shown among *BRCA1* or *BRCA1/2* mutation heterozygotes but not among *BRCA2* mutation carriers only, compared to non-carriers, and it may have contribution to the improved prognosis (Husain et al. 1998, Cass et al. 2003). Patients in the present study received cisplatinum-based

chemotherapy for ovarian cancer, and one patient received also chemotherapy including mitoxantrone (CMF/CNF) for breast cancer prior to the ovarian cancer. Thus, it can be speculated that the mutation enhances the response to such chemotherapy, improving prognosis.

There is clearly need for collection of data on the effect of individual mutations, as well as of response to treatment among mutation carriers. The 4088insA mutation provides an example of how the knowledge of the effect of an individual mutation may be important in estimating prognosis for mutation carriers. The mutation in a breast/ovarian cancer family may be of auspicious nature and the knowledge of this may provide some relief in the distress for the family members carrying the mutation. In the near future the genetic information of the germ-line of the cancer patient may be important in assessing prognosis of the disease on a wider scale.

6.2 Sporadic breast cancer and new low-penetrance susceptibility genes in Eastern Finland (IV, V)

6.2.1 Autosome-wide scan for LD-based breast cancer association (IV)

Our scan was conducted in order to gain knowledge of possible genetic breast cancer risk factors on population level. We selected the cases that did not have a strong family history of breast cancer from a small geographical region for optimal homogeneous gene pool and as small effective number of unrelated founders as possible (Kruglyak 1999). Also when the cases are a homogeneous group from small geographical region the subset of genetic risk factors they carry could be also homogeneous and the number of them reduced. We aimed at identifying low-penetrance risk factors for breast cancer which does not show up clearly in families. The genetic diversity of this population is believed to be lower than that of the Finnish population in general and this feature has been the basis for mapping several genes and even genes for complex diseases (Ekelund et al. 2000). Due to the nature of our population this study falls in between traditional LD study and family linkage study. Being a young isolated population this group of Eastern Finns could be seen as a sample set in between a family set and a group of non-relatives. When this work was initiated (1996) it was not recognised as it is according to

the current knowledge that it could be useful to have family material even in association studies (Antoniou and Easton 2003). Also, at the time of the sample selection the contribution of high-penetrance genes, e.g. *BRCA1* and *BRCA2*, was not known and we threfore chose non-familial breast cancer cases in order to identify low-penetrance susceptibility genes. Very recently though, it has been shown that the same low-penetrance risk factors could be identified in a sample set of sporadic breast cancer and among cases with a strong family history of breast cancer (Hunter et al. 2007, Easton et al. 2007).

Of the 366 markers in the first-stage scan 59 % were spaced by ≤10 cM, 30 % by 11-15 cM and 11 % were spaced by 16-20 cM. Less than half of the screened chromosomes harboured one or two gaps of 18-20 cM. However, in this approximately 20 generations old founder population relatively few meioses have occurred and linkage disequilibrium should extend beyond the distances between adjacent markers in the first and second-stage scans in general (Ophoff et al. 2002). In a sub-isolate of Kuusamo in the late-settlement region up to 50 % of microsatellites showed LD at distances 3.5-7.5 cM and 30 % at distances >7.5 cM (Varilo et al. 2003). Our study population was selected from a small rural area in the late-settlement region and could possess the genetic characteristics more similar to the Kuusamo population than the late-settlement population in general. It is also worth noting that with a 10-cM map LD between marker and risk allele needs to extend 5 cM, as our scan is not for LD between markers but between marker and risk allele that lies somewhere between two markers spaced by 10 cM in average. LD between SNP markers extends a much shorter distance. A study comparing the extent of LD between SNP markers with minor allele frequency >0.1 in four populations of European ancestry but different demographic histories, Ashkenazim, Africaners, East Anglian British and Eastern Finns, found that the average LD was similar in all populations (Dunning et al. 2000). However, there are regions in the genome where the LD varies between populations, e.g. the Eastern Finnish population showed somewhat higher LD in regions on chromosome 22 (Dunning et al. 2000). Also, in the Eastern Finnish poulation LD was detected at marker pairs 200-500 kb apart while the other three studied populations did not show LD different from the baseline at these distances (Dunning et al. 2000).

6.2.1.1 Candidate-genes for further studies on breast cancer association (IV)

In the present study altogether 27 markers in 16 chromosomes showed association with breast cancer in the two-staged autosome-wide scan using 435 microsatellite markers. In the haplotype analysis three chromosomal regions, 3p26, 11q23 and 22q12-q13 were further suggested as candidate locations for breast cancer-associated genes.

Testing 366 markers at the 0.05 significance level is predicted to give 18 positive associations by chance alone (366x0.05). Since we found 21 positives in the first stage, it is reasonable to propose that some of these represent true associations. Haplotype analysis using global-score method and markers in and around associated chromosomal sites revealed four positive regions, which fits well with this assumption. Estimating the number of possible false positive results in the second-stage scan is more complicated because the markers were selected next to the markers that are in LD with breast cancer and therefore are not independent. The Bonferroni correction was not used here as it is very conservative and we view these results as a guide for further investigation and not statistically definite significant associations. Thus we wanted to follow up all possible associations in the second-stage scan. Also, it is unclear how the adjusting for multiple comparisons should be best done in genome-wide screenings (Ophoff et al. 2002) and a method that identifies false positives has not been introduced. When studying complex diseases it is expected that there will be multiple loci affecting the trait and a very strict control of the number of false positives (such as the Bonferroni method) will result in a considerable loss of power to identify secondary signals (Ophoff et al. 2002).

We are aware that all possible breast cancer-associated loci may not have been recognised in our screen due to the longer distance between markers in some chromosomal regions and a limited sample set. Also, at the time when this scan was initiated the exact physical locations of some markers were not available. As a consequence some markers used in the second-stage scan are not as close to markers that were in LD in the first-stage scan as initially expected. This, in turn, could lead to false negative results. Therefore, a denser marker map and a bigger sample set could probably have led to an improved result of the scan. Considering all that, it may not be best to limit the search in the three chromosomal regions, 3p26, 11q23 and 22q12-q13, identified in the haplotype analysis but to investigate possible candidate-genes near all

individual markers that showed breast cancer association. Some of the genes are proposed to be cancer-related and some of them could be candidates for breast cancer-associated genes based on the proposed function of the gene product. In the 560-kb haplotype region on 3p26 there are three genes, two of which are not very well characterised or properly named. Evidence from loss of heterozygosity (LOH) studies suggests a few candidate tumour suppressor genes lie on chromosome 3p (Yang et al. 2002c) but none of them locates in the 560-kb region of the significant haplotype found in our scan. Interestingly though, a linkage analysis conducted on Finnish prostate cancer families identified 3p26 as a locus for hereditary prostate cancer gene (Schleutker et al. 2003, Rökman et al. 2005). On 11q23 the 900-kb haplotype region includes 13 named genes and four hypothetical genes. Chromosome 11q21-24 region is also known for LOH in several different cancers including breast cancer. In the 550-kb region of the two significant haplotypes on 22q13.1 locates seven named genes, two non-characterised and two hypothetical genes. Another 16 interesting genes locate near the markers with breast cancer association in other chromosomes.

Recently three genome-wide association studies for breast cancer risk alleles have been conducted (Easton et al. 2007, Stacey et al. 2007, Hunter at el. 2007). Two of them used sporadic case-control material (Hunter et al. 2007, Stacey et al. 2007), whereas in the third GWS case material selected for a strong family history of breast cancer was used in the initial scan (Easton et al. 2007). Regardless, these GWSs identified the strongest associations to *FGFR2* (Easton et al. 2007, Hunter et al. 2007) and *TNRC9* (Easton et al. 2007, Stacey et al. 2007) genes, although each of them identified also additional associations that were not detected in other studies. This indicates that low-penetrance risk factors for breast cancer can be detected using GWS. On the other hand even the new-generation GWSs using SNPs do not find all risk factors and the identification of the causal variant can be troublesome. In our microsatellite scan we detected 27 positive associations. One of these (*D2S434*) locates near to (670 kb) the chromosome 2q35 association found in the GWS conducted by Stacey and coworkers (2007). A borderline significant (P=0.95) marker (*D10S1213*) and an associated allele with it was detected 2 Mb from the described *FGFR2* association, and only 400 kb from the other associated SNP on 10q (rs10510126). Clearly though, it can not be argued to

be the same association without further studies. Other associations detected in our scan were not in the vicinity of the associations described in the more recent GWSs. However, more associations were detected than expected by chance alone and some of these can represent true risk factors. Thus, our scan is also a feasible method for initial screen for identification of potential candidate regions for follow-up studies.

6.2.2 Breast cancer association on chromosome 22q12-q13 (V)

6.2.2.1 *TMPRSS6* as a candidate-gene (V)

In the present study, the most promising breast cancer association was detected with a SNP rs733655 in *TMPRSS6* gene. We observed an increased risk associating with the heterozygous genotype TC of rs733655 and with the rare allele C carriers but not at the homozygous genotype CC carriers. This may be partly explained by too small number of observed rare homozygotes alone to show significant increase in risk and a larger sample set might solve this problem.

In the controls the rs733655 genotypes deviated slightly from HWE whereas the cases were in equilibrium. However, in the RefSNP European test population sample the HWE probability is 0.05 indicating that in the normal situation the genotype frequencies deviate from those expected under HWE (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=733655). The rs733655 allele frequencies that we observed among controls are also similar to those expected in the NCBI RefSNP database. The allele frequencies among our cases differ from those expected (RefSNP) as genotypes that we observed do not deviate from HWE. If the number of controls by genotype is replaced by the numbers expected under HWE, the difference in genotype frequencies between cases and controls still remains significant in the subsample (which includes only cases born in the province of Northern Savo and their age and long-term area-of-residence matched controls) (P=0.013). Also, the risk for the heterozygous genotype TC remains significant in the subsample, although reducing from 2.11 to 1.67 (CI 1.17-2.39). When validating the SNPs for this study we sequenced 20 samples for rs733655 and no sequence variation other than rs733655 was observed in the region of the designed TaqMan primer and probe sequences.

Genotyping assays were optimized and allele calling was unambiguous as allele controls were used. Also 141 samples from altogether six plates were re-genotyped, confirming the original genotype and thus, a genotyping error is also excluded. Therefore, we consider that the detected difference between the case and control populations is real. Although it is common to exclude markers which deviate from HWE from association studies it is possible to miss true risk factors by doing so. When genotyping artefacts are excluded it leaves open the possibility that common variants that show departure from HWE are subject to selection, acting either directly or indirectly via selective sweep accross other variants in LD with them (Baynes et al. 2007).

TMPRSS6 gene encodes a membrane-bound serine proteinase 6 called matriptase-2. Matriptase-2 is a member of a family of type II transmembrane serine proteinases which have possible role in cancer development. Matriptase-2 has the ability to degrade extracellular matrix components, suggesting that it may participate in some of the matrix-degrading processes occurring in both normal and pathological conditions, including cancer progression (Velasco et al. 2002, Netzel-Arnett et al. 2003). As matriptase-2 is only recently discovered, little is known about the physiological function. Matriptase-2 is expressed in normal breast tissue and the expression is elevated in breast cancer (invasive ductal carcinoma) (Overall et al. 2004). *TMPRSS6* has also been detected to be mutated in breast carcinomas (Sjöblom et al. 2006). Thus, the possibility of matriptase-2 involvement in cancer progression is supported and *TMPRSS6* is a potential novel candidate for breast cancer risk affecting gene. More specific investigation for the association between matriptase-2 and breast cancer, as well as the identification of the causal genetic variant is needed. To resolve the global importance of *TMPRSS6* in genetic risk of breast cancer the association has to be tested in other populations also.

According to the power calculations our sample set of 497 cases and 458 controls had >95 % power to detect a risk allele that is in perfect LD with the marker allele (D'=1) and has a relative risk of 2. The power to detect a risk allele that is not in perfect LD with the marker allele (D'=0.5) and has a relative risk of 2 varies between 44-74 % with the ten SNPs. In the stratified sample the power for the detected risk (2.11 for the

TC and 1.16 for TT genotype) with SNP rs733655 is 83 %. Within a short distance (1 kb) we detected highly significant LD (D'=1) and even at distances longer than 200 kb D' is ≥ 0.5 at some instances. It is presumable that LD does not remain at the constant level across the whole 516-kb region and some associations may therefore have been missed.

Our negative results do not rule out association involving other nearby SNPs and positive results do not necessarily indicate the discovery of the causal SNP but a marker in LD with a true causal SNP located some distance away.

6.2.2.2 Other genes on chromosome region 22q12-q13 (V)

Another SNP, rs7285064 in *CSF2RB* gene, showed moderate association with breast cancer. In the subsample the heterozygous genotype associated with a reduced risk of breast cancer indicating a protective effect. Further evidence concerning the importance of this gene in breast cancer is necessary. Other genes in the studied region have not been reported to associate with (breast) cancer.

6.2.2.3 Allelic imbalance on chromosome region 22q12-q13 (V)

Matriptase-2 is expressed in normal breast tissue and the expression is elevated in breast cancer (invasive ductal carcinoma) (Overall et al. 2004), which points out that matriptase-2 is expected not to be a tumor suppressor. Our AI analysis provided further support to this since AI was not detected in the vicinity of *TMPRSS6* gene. This also indicates that the detected AI seems to be a separate event from the original breast cancer association, i.e. hereditary risk factor, while AI may indicate the involvement of a nonhereditary factor. However, in line with other studies (Allione et al. 1998, Iida et al. 1998, Bryan et al. 2000, Castells et al. 2000, Hirano et al. 2001) the observed AI on the 22q12-q13 region does not exclude existence of a tumor suppressor, other than *TMPRSS6*.

7. SUMMARY AND CONCLUSIONS

Breast cancer is the most common cancer in women and ovarian cancer is also among the most common cancers. The objective of this study was to study the contribution of known high-risk breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, in Eastern Finnish families. The second objective was to find chromosomal regions and genes in them that associate with the risk of (sporadic) breast cancer.

This study demonstrated that:

1. Germ-line mutations in *BRCA1* and *BRCA2* genes account for 19.4 % of the studied Eastern Finnish breast/ovarian cancer families. The observed Eastern Finnish mutation spectrum differs from those observed in the Northern and Southern parts of the country. Only two of the eight most common Finnish founder mutations were detected in the Eastern Finnish families. In addition, one of the mutations has not been found elsewhere in Finland (*BRCA2* 4088insA). The results add to the evidence of the geographical differences in distribution of Finnish *BRCA1* and *BRCA2* mutations. This screen also provides further evidence for the presumption that the majority of Finnish *BRCA1*/*BRCA2* founder mutations have been found and that the proportion of *BRCA1*/*BRCA2* mutations in Finnish breast/ovarian cancer families is around 20 %.

2. A *BRCA2* mutation 4088insA, novel to Finland, was found in one family of Eastern Finnish origin. The mutation is highly penetrant; four of the six carriers are affected with breast and ovarian, bilateral breast or bilateral ovarian cancer and one with prostate cancer. Despite of the high penetrance this mutation seems to be associated with better than expected outcome. The mutation positive breast/ovarian cancer patients in this family had an excellent response to treatment even when prognosis assessed by classical factors was poor.

3. The *BRCA1* exon 12 4216-2nt A→G substitution mutation was confirmed to disrupt the splice acceptor dinucleotide AG and to form another splice acceptor

dinucleotide. Wrong splicing site leads to inclusion of an extra nucleotide and a frameshift in translation that causes a premature translation termination and a truncated protein product.

4. In the first stage of the autosome-wide linkage disequilibrium-based screen 21 STR markers on 16 chromosomes showed initial association with breast cancer. A further analysis with additional markers and haplotypes restricted breast cancer associations to three chromosomal regions, 3p26, 11q23 and 22q13.

5. A 516-kb region on chromosome 22q12-q13 was further refined using 10 SNP markers. Significant association was detected with one SNP located in the intronic sequence of *TMPRSS6* gene encoding matriptase-2. The heterozygous genotype and the minor allele were associated with increased risk of breast cancer. In the AI analysis of 22q12-q13 abundant AI was detected more centromeric than previously reported, further supporting the existence of a tumour suppressor gene or genes in this region.

In conclusion, mutations detected in the known high-risk and high-penetrance breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, account for approximately 20 % of the familial breast/ovarian cancer in Eastern Finland. This corresponds to the observed contribution of these genes in families in other parts of our country. In our search for additional breast cancer risk associated genes we have found an interesting new candidate, *TMPRSS6*, which warrants additional association and functional studies. In addition, chromosomal regions 3p26 and 11q23 may harbour breast cancer-associated genes.

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9. ORIGINAL PUBLICATIONS

Kuopio University Publications D. Medical Sciences

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